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Marine organisms living in environments containing toxic chemicals are often exposed simultaneously to many different classes of compounds, which collectively pose a different threat of toxicological effects than is posed separately by the individual compounds. The present research was directed toward elucidating the effect of xenobiotics which alter the metabolism and toxicity of aromatic hydrocarbons by marine fish (salmon and flounder), as evinced through biochemical changes and altered cellular morphology. The xenobiotics used included petroleum aromatic hydrocarbons, chlorinated biphenyls, p-cresol, cadmium and lead.

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FOREWORD

Along with the accelerated development of petroleum resources on the continental shelf of the United States and continued importation of petroleum of foreign origin, we can expect increased transfer and refinement activities in coastal areas, with an associated incidence of oil pollution. In order to develop an adequate understanding of the potential marine environmental consequences of such pollution, the National Oceanic and Atmospheric Administration has conducted studies on the Fate and Effects of Petroleum Hydrocarbons and Toxic Metals in Selected Marine Organisms and Ecosystems under Interagency Agreement with the Environmental Protection Agency. The overall objectives of this effort have been to study experimentally specific processes underlying the distribution, transport and biological effects of petroleum hydrocarbons in coastal marine ecosystems. The results are expected to facilitate the assessment of impacts of petroleum releases, and thereby to improve the basis for developing regulatory measures for suitable protection of the marine environment. A primary concern expressed consistently during early considerations of study priorities was that different classes of xenobiotic compounds, introduced into the environment as a result of various human activities, might act synergistically to produce effects of greater magnitude than would be predicted from experimental results with individual compounds. The report that follows presents results directed at this experimentally difficult area of concern.

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ABSTRACT

Marine organisms living in environments containing toxic chemicals are often exposed simultaneously to many different classes of compounds, which collectively pose a different threat of toxicological effects than is posed separately by the individual compounds. The present research was directed toward elucidating the effect of xenobiotics which alter the metabolism and toxicity of aromatic hydrocarbons by marine fish, as evinced through biochemical changes and altered cellular morphology. The xenobiotics used included petroleum aromatic hydrocarbons, chlorinated biphenyls, p-cresol, cadmium and lead.

Coho salmon (*Oncorhynchus kisutch*), maintained in seawater at 7°C and fed model mixtures containing chlorobiphenyls, petroleum hydrocarbons, and a mixture of the two classes of compounds, were examined for uptake of these chemicals in liver, kidney, and residual body tissues comprised of eviscerated, headless and tailless carcass; and for possible changes in the activities of hepatic microsomal aryl hydrocarbon hydroxylase (AHH). Analyses of the coho salmon tissues indicated that the concentrations of individual chlorobiphenyls were highly variable; the concentrations of the hydrocarbons were below detection limits, suggesting that they were metabolized and/or excreted by the fish. In addition, the activities of hepatic AHH were induced by mixtures of the chlorobiphenyls and petroleum hydrocarbons, but not by the chlorobiphenyls alone or hydrocarbons alone. An apparent synergism, reflected by increased AHH activities, occurred when the chlorobiphenyls and hydrocarbons were administered together.

Chinook salmon (*Oncorhynchus tshawytscha*), maintained in seawater at 13°C and fed mixtures of chlorobiphenyls and petroleum hydrocarbons separately and together, were examined for changes in hepatic microsomal AHH activity and for alterations in the morphology of liver, kidney, intestine, gill and skin tissues. The data suggested that the AHH activity was affected differently for chinook than for coho salmon. Using chinook salmon microsomes, AHH activities were found depressed for all fish treated with either the chlorobiphenyls or the hydrocarbon mixture. In addition, morphological changes relating to inclusions in the cells of the intestinal mucosa were observed for chinook salmon fed either hydrocarbons or chlorobiphenyls; whereas, considerable sloughing of the mucosal epithelium occurred in fish treated with the combined mixtures. The latter finding also indicated an interactive effect of the two classes of xenobiotics. Additional alterations were found in some hepatocytes, but not in the other tissues.

The differences for the coho and chinook salmon in terms of the responses of the hepatic AHH systems to petroleum hydrocarbons and chlorobiphenyls may have been caused by differences due to seasonal parameters between the two experiments. Hence, depending on the temperatures, normal biotransformations of petroleum in exposed marine

organisms may be enhanced or retarded, assuming no species differences. These xenobiotic effects may be influential factors in marine environments.

Coho salmon and starry flounder (Platichthys stellatus), exposed to 200 ppb of cadmium or lead in seawater at 10°C, were fed a model mixture of polycyclic aromatic hydrocarbons (PAH's) consisting of 2-methylnaphthalene, 2,6-dimethylnaphthalene, and phenanthrene. The effects of the metals were determined on the AHH activity of liver microsomes. (Concentrations of cadmium and lead in livers were not determined.) The results suggest that hepatic hydroxylations of PAH's, using naphthalene as a substrate, are not affected by exposure of fish to these metals at 200 ppb. Addition of 4-5 ppm cadmium to reaction mixtures containing liver microsomes caused 82-98% inhibition of AHH activity; lead at 10 ppm caused 84% inhibition. Thus, the possibility exists that alterations in PAH metabolism may occur if cadmium or lead accumulates in salmon and flounder livers. Such alterations may result in changes in the toxic effects of PAH's in organisms exposed to petroleum in the environment.

Benzo[a]pyrene, 2,6-dimethylnaphthalene, and naphthalene were used as substrates for comparisons of AHH activities in a preparation from coho salmon liver microsomes. The apparent Michaelis constants (Km; the concentration of substrate required to obtain one-half of the maximum reaction velocity with a given concentration of enzyme) were determined, and the results indicated that salmon microsomal mixed-function oxidase systems have a higher affinity for benzo[a]pyrene and 2,6-dimethylnaphthalene than for naphthalene. Because results indicate that microsomal preparations from livers have a high affinity for 2,6-dimethylnaphthalene, and that the latter compound is more soluble in water and less hazardous to handle than is benzo(a)pyrene, 2,6-dimethylnaphthalene is a useful substrate for research on evaluating effects and dispositions of xenobiotics introduced into marine environments through petroleum-related activities.

Microbial tests for mutagenicity were performed on 2,6-dimethylnaphthalene, on four oxygenated derivatives of 2,6-dimethylnaphthalene, and on the naphthalene derivative, trans-1,2-dihydroxy-1,2-dihydronaphthalene. All tests for mutagenicity were negative; however, 2,6-dimethyl-3,4-naphthoquinone was lethal to the test organism, Salmonella typhimurium. As far as can be generalized from microbial bioassays, mutagenic reactions are unlikely in fish due to exposures to these naphthalenic chemicals.

The hepatic microsomes from coho salmon were used in the elucidation of the metabolism of 2,6-dimethylnaphthalene. Major metabolites produced were 6-methyl-2-naphthaldehyde and 6-methyl-2-naphthalenemethanol. Products of naphthyl-ring oxidations included a quinone, two naphthols, and a dihydrodiol of 2,6-dimethylnaphthalene. Studies of PAH metabolism in vitro provided a basis for preparing metabolites for chemical analyses, which are necessary for elucidating the metabolic fate of PAH's in marine organisms in this and future work.

Other studies were performed to determine the effects of exposing starry flounder to naphthalene, p-cresol, or a mixture of both compounds. The work included (1) in vivo synthesis of metabolites from 2,6-dimethylnaphthalene, and (2) the biosynthesis of lipids. Biliary metabolites of 2,6-dimethylnaphthalene consisted primarily of conjugates, namely, glucuronides and glucosides. Exposures of flounder to naphthalene, p-cresol, or both indicated a significant reduction of the glucuronide of 6-methyl-2-naphthalenemethanol in the bile and a corresponding increase in the glucuronide of trans-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene. Such a shift in metabolite composition, from products of methyl-group oxidation to those of aromatic-ring oxidation, may result in metabolites which are more toxic.

In the study of xenobiotic-induced alterations in lipid biosynthesis, significant decreases were observed in the incorporation of acetate into free fatty acids in liver of flounder exposed to naphththalene alone ($P \leq 0.05$) or together with p-cresol ($P \leq 0.01$), compared to that of controls. Free fatty acid synthesis was apparently not altered upon exposure of animals to p-cresol alone; however, all exposure groups experienced changes in the biosynthesis of triglycerides, as reflected by decreases in the incorporation of oleate into triglycerides. No evidence was found to indicate that phospholipid biosynthesis was altered. Because triglycerides are a source of energy for fish, xenobiotic-induced alterations in biosynthesis of these compounds may pose a threat to the viability of exposed fish.

A study of the effects of 2,6-dimethylnaphthalene and p-cresol on liver-cell morphology of coho salmon has established that these chemicals, separately or together at 10-20 ppm, damaged the sinusoids and certain organelles of the hepatocytes. The morphological changes, particularly those involving the sinusoids, are similar to hepatic changes seen in a variety of other animals afflicted with conditions such as hepatitis and toxic-chemical injury.

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SECTION 1

INTRODUCTION

The toxicity of petroleum or pretroleum products toward marine animals will result from the combined effects of a complex mixture of organic and inorganic chemicals. Observed toxic effects may not be simply a sum of the effects of the individual components, but rather the influence of interactions between different chemicals.

Although it is important to assess the toxicity of single chemicals, interactive effects cannot be inferred from such research. Direct chemical interactions between xenobiotics may give rise to new chemicals that will have greatly enhanced or reduced toxicities. Also, in vivo potentiation, addition, or antagonism may result from simultaneous exposure to mixtures of xenobiotics and their metabolites (Conney and Burns, 1972; Krieger and Lee, 1973; Lichtenstein et al., 1973; Livingston et al., 1974; O'Brien, 1967). Most laboratory investigations have involved studies of accumulation, depuration, and toxicity of individual classes of contaminants. This is particularly true for chlorobiphenyls, petroleum-related hydrocarbons, and heavy metals (Hansen, et al., 1971; Malins, 1977, Stalling and Mayer, 1972; Waldichuk, 1974). Prior to this research, there was little information on the combined effects of chlorobiphenyls and polycyclic aromatic hydrocarbons (PAH's) on marine fish. Interactive effects of mixtures of chlorobiphenyls and PAH's or of mixtures of PAH's had not been assessed in marine organisms prior to the preliminary work by Gruger et al. (1977a).

Fish possess xenobiotic-metabolizing enzyme systems which mediate oxidations of compounds such as aromatic hydrocarbons (Adamson, 1967; Ahokas et al., 1975; Buhler, 1966; Buhler and Rasmusson, 1968; Pohl et al., 1974). These enzyme systems, present in microsomes prepared from liver at usually higher concentrations than from other tissues, have served as an index for evaluating potential toxicities of xenobiotics in various animals (Gelboin et al., 1970; Franke, 1973). In addition, the activities of these enzymes from fish have been proposed as indicators of pollution of aquatic environments (Ahokas et al., 1976; Payne, 1976 Payne and Penrose, 1975). In other words, when fish are exposed to, for example, PAH's from petroleum, the activities of the liver enzymes, aryl hydrocarbon hydroxylases (AHH), are useful in evaluating changes that may occur in the metabolism and toxicity of the PAH's (Kurelec et al., 1979).

The principal objective of the present research, between 1975 and 1978, was to provide information about the effects of biochemical interactions of chlorinated hydrocarbons and PAH's on the accumulation, metabolism, and potential toxicities of PAH's in marine fish. The objective was expanded in 1977 to include information about interactive effects of heavy metals on the metabolism of PAH's. From 1978 to October 1980, the work was further expanded to provide information about possible interactive effects of two xenobiotics on the metabolism of a third xenobiotic, namely, the combined effects of a representative PAH and an oxygenated PAH on the metabolism of an alkyl-substituted PAH. In addition, a study was conducted on possible effects of the two former classes of compounds on lipid biosynthesis in fish. Related studies of possible alterations in cellular morphology of experimental fish were also an integral part of the research.

The research had the following specific objectives over the five year period: (1) To determine changes that may occur in the accumulation, metabolism, and disposition of mixtures of chlorobiphenyls and PAH's in target tissues of coho salmon (*Oncorhynchus kisutch*) exposed to those compounds singly and together in food; (2) to determine the effects of mixtures of chlorobiphenyls and PAH's in coho salmon and chinook salmon (*Oncorhynchus tshawytscha*) on the activities of hepatic microsomal AHH; (3) to determine whether those mixtures cause possible alterations in gross pathology and cellular morphology of salmon; (4) to determine possible effects of a mixture of PAH's and cadmium or lead in marine fish on the activities of hepatic AHH; (5) to determine and compare activities of AHH from coho salmon liver microsomes toward three PAH's (i.e., naphthalene, 2,6-dimethylnaphthalene, and benzo[a]-pyrene); (6) to elucidate the *in vitro* metabolism of 2,6-dimethylnaphthalene by coho salmon liver microsomes; (7) to synthesize a series of oxygenated derivatives of 2,6-dimethylnaphthalene for use as analytical standards and for tests of mutagenicity; (8) to elucidate the effects of naphthalene and p-cresol, singly or together, on the metabolism of 2,6-dimethylnaphthalene in starry flounder (*Platichthys stellatus*); (9) to determine whether 2,6-dimethylnaphthalene and p-cresol, singly or together in coho salmon, cause alterations in the cellular morphology; and (10) to determine whether naphthalene and p-cresol, singly or together in starry flounder, alter lipid biosynthesis.

SECTION 2

CONCLUSIONS

The findings from this study lead to the following conclusions:

1. Simultaneous exposure of salmonids to chlorinated biphenyls and aromatic hydrocarbons significantly influence the biotransformations of aromatic hydrocarbons. In chinook salmon, exposures to chlorinated biphenyls and aromatic hydrocarbons alone also cause alterations in metabolism of aromatic hydrocarbons. These interactions of xenobiotics appear to be influential in modifying toxic effects in marine organisms exposed to petroleum.

2. Exfoliation of intestinal epithelium can occur in chinook salmon exposed to chlorinated biphenyls or to a mixture of petroleum hydrocarbons and chlorinated biphenyls; the combined effect of the two types of xenobiotics appears to be much greater than with the chlorobiphenyls alone, suggesting an interaction. These compounds singly or together had no effect on the morphology of liver, kidney, gills or skin from exposed chinook salmon.

3. In coho salmon and starry flounder, no marked alterations occur in metabolism of polycyclic aromatic hydrocarbons (PAH's) when fish are exposed to waterborne cadmium or lead for prolonged periods. However, alterations may result if very high concentrations of these metals accumulate in livers.

4. The aryl hydrocarbon monooxygenase system of coho salmon liver microsomes has an affinity for 2,6-dimethylnaphthalene that is near to that for benzo[a]pyrene and much greater than that for naphthalene. 2,6-Dimethylnaphthalene is a convenient and useful compound for evaluating the biochemical fate and effects of low-molecular weight alkyl-substituted PAH's from petroleum.

5. Mutagenic reactions, as far as can be generalized from microbial bioassays, are unlikely in fish due to exposures to 2,6-dimethylnaphthalene, 6-methyl-2-naphthalenemethanol, trans-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene, trans-1,2-dihydroxy-1,2-dihydronaphthalene, 2,6-dimethyl-3-naphthol or 2,6-dimethyl-3,4-naphthoquinone.

6. The composition of metabolites of 2,6-dimethylnaphthalene shifts from products of alkyl-group oxidation to naphthyl-ring oxidation in starry flounder exposed to naphthalene, p-cresol, or both xenobiotics together. Such a shift in metabolism of alkyl-substituted PAH's implies that different toxicities of these compounds may occur in fish exposed to mixtures of different xenobiotics.

7. Triglyceride biosynthesis is altered in starry flounder exposed to naphthalene, p-cresol, or a mixture of the two xenobiotics. In addition, biosynthesis of fatty acids is altered in flounder exposed to naphthalene with or without p-cresol. Such xenobiotic-induced changes in the biosynthesis of neutral lipids may pose a threat to the viability of exposed fish which must rely on lipids as energy sources and for other essential physiological functions.

8. Alterations were observed in the morphology of hepatocytes of coho salmon exposed to 2,6-dimethylnaphthalene and p-cresol. These alterations, indicating toxic-chemical injury, would predictably be harmful to fish exposed to such compounds from petroleum pollution.

SECTION 3

RECOMMENDATIONS

The findings from this research suggest that exposure of marine fish to mixtures of xenobiotics produces quite different results at both the biochemical and morphological levels from results obtained from exposures to the single compounds of the mixtures. Clearly, more work is needed to examine the effects of combinations of xenobiotics. For example, additional research is needed to elucidate to what extent chlorinated hydrocarbons, in a wide range of concentrations in the environment, can possibly change the composition of metabolites of PAH's and alter toxicities in different marine species. Eventually, every effort should be directed toward a better understanding of the combined effects of multiple contaminant systems characteristic of polluted marine environments.

Future studies of the combined effects could include areas of marine biology such as predator-prey relationships, reproductive behavior and success, and feeding behavior. Studies of these kinds should be supported by an interdisciplinary approach involving physiology, morphology, biochemistry, and analytical chemistry.

SECTION 4

EXPERIMENTATION

EFFECTS OF CHLORINATED BIPHENYLS AND PETROLEUM HYDROCARBONS ON THE UPTAKE AND METABOLISM OF AROMATIC HYDROCARBONS IN SALMONIDS

Materials

Selection of Experimental Fish--

Approximately 2,400 coho salmon were obtained from seawater facilities of Domsea Farms, Gorst, Washington. Groups of 20-30 fish were anesthesized with tricaine methanesulfonate (MS-222) and their lengths and weights were measured. After the weighings, the fish were distributed and maintained in outdoor floating net pens. The pens were located in seawater at an aquaculture facility of the Northwest and Alaska Fisheries Center on Puget Sound, near Manchester, Washington. Each pen (4 ft x 7 ft x 6 ft deep with 1.5 ft above water) contained 150 fish. For these experiments, 2,100 fish were held in 14 pens for duplicate exposures. The initial range of mean weights ±S.D. for the 14 groups was 184±41 to 204±41 g.

Approximately 600 chinook salmon were obtained from the aquaculture facility of the Northwest and Alaska Fisheries Center. These fish were treated in the same manner as the coho salmon, but were distributed in four net pens in seawater. The initial weights of chinook salmon in the four groups ranged from 72±14 g to 76±16 g.

Preparation of Control and Test Diets--

The basal diet for all fish was Oregon moist pellets (Hublou, 1963), which were prepared from ingredients obtained from Moore-Clark Co., La Conner, Washington. In order to minimize extraneous organic contaminants such as PCB's in the diet, a fish protein concentrate (25.2% wt/wt) was substituted for herring meal and extra food-grade soybean oil was added to the preparations. The basal diet served as the control diet. Test diets were prepared from the basal diet with chlorobiphenyls and hydrocarbons added as separate mixtures in soybean oil solutions. For coho salmon fed diets with chlorobiphenyls, the mixture of chlorobiphenyls consisted of 17.8% (wt/wt) biphenyl, 11.6% 2-chlorobiphenyl, 19.8% 2,2'-dichlorobiphenyl, 9.6% 2,4'-dichlorobiphenyl, 23.8% 2,5,2'-trichlorobiphenyl, and 17.3% 2,5,2',5'-tetrachlorobiphenyl. For chlorobiphenyl diets fed to chinook salmon, the mixture of chlorobiphenyls consisted of 20.2% biphenyl, 19.1% 2-chlorobiphenyl, 20.9% 2,2'-dichlorobiphenyl, 20.2% 2,5,2'-trichlorobiphenyl, and 19.8% 2,5,2',5'-tetrachlorobiphenyl. Coho salmon were fed a hydrocarbon mixture that consisted of 12.1% 2,3-benzothiophene, 9.8% n-pentadecane, 12.2% 2,6-dimethylnaphthalene, 12.6% 2,3,6-trimethylnaphthalene, 12.6% fluorene, 12.7% 1-phenyldodecane, 12.4% phenanthrene, and 15.5% heptadecylcyclohexane. Chinook salmon were fed a mixture of hydrocarbons that consisted of 14.8%

benzothiophene, 12.6% 2-methylnaphthalene, 10.9% 2,6-dimethylnaphthalene, 11.4% 2,3,6-trimethylnaphthalene, 10.7% 1-phenyldodecane, 14.1% fluorene, 13.4% phenanthrene, and 12.1% heptadecylcyclohexane. The chlorobiphenyls (Analabs Inc., North Haven, Conn.; RFR Corp., Hope, Rhode Is.) and the hydrocarbons (Chemical Samples Co., Columbus, Ohio; Chem. Service, Inc., Westchester, Penn.; Aldrich Chemical Co., Inc., Milwaukee, Wis.) were obtained from commercial sources and used without further purifications.

The pelleted diets were prepared in lots of 4.5 kg, using aliquots (10 g and 50 g) of stock solutions of mixtures of the test compounds in food-grade soybean oil. The test diets consisted of 1 and 5 ppm (wt/wt) of chlorobiphenyls, 1 and 5 ppm of hydrocarbons, and 2 and 10 ppm of 50-50 mixtures of the two types of compounds. The pellets were stored at -15°C until used.

Procedures

Feeding Schedules--

Fish were acclimated for three weeks before the initiation of the exposure experiments. Coho salmon were fed 1 ppm and 5 ppm hydrocarbons and chlorobiphenyls alone. In addition, coho salmon designated to receive the 2 and 10 ppm of the 50-50 mixtures of xenocompounds were fed the diets containing 1 and 5 ppm of chlorobiphenyls, respectively, for one week before beginning the feeding of the mixtures. The coho were fed three to four times daily at a daily rate of 0.75% of the biomass, which was adjusted for the weights of fish removed from the pens for analysis. (Examination of stomach contents revealed food pellets, indicating that the food was ingested.) The experiments were carried out from February to April, 1976, when ambient seawater temperature was low (7°C), so that a feeding rate of 0.75% of biomass was a maintenance ration. Test coho salmon were fed their respective diets for four weeks, followed by four weeks with control diets.

The four groups of chinook salmon were fed the control diet, and diets containing 5 ppm chlorobiphenyls, 5 ppm hydrocarbons, and 10 ppm of the 50-50 mixtures of chlorobiphenyls and hydrocarbons, respectively. The experiment with the chinook salmon was performed during July and August, 1976, when ambient seawater temperature was about 6°C greater (13°C) than during the forementioned experiments with coho salmon. In a manner of feeding similar to that for coho salmon, the chinooks were fed twice daily at a rate of 2.5% of the biomass per day; that rate was chosen to provide a minimum growth to the fish during July and August. Test fish were fed diets containing the xenocompounds for four weeks, followed by a return to control diets for the remainder of the time.

Handling of Samples--

Ten coho salmon for AHH analyses and six coho for chemical uptake analyses were taken from half of duplicate exposure groups at weekly intervals, alternating between duplicates. Only ten chinook salmon were taken periodically from each group for AHH analyses. In addition, fish were taken for microscopic examinations with sampling and handling protocols different than for chemical and AHH analyses (cf., below). Fish were sacrified by cervical dislocation, and lengths and weights were measured. For chemical analyses, fish were quickly placed over dry ice and transferred to laboratory storage at -60°C. For AHH analyses, livers were excised, sex determined, and inspections were made for abnormalities in physical characteristics. Excised livers were rinsed with chilled 0.25 M-sucrose, immediately placed into screw-capped vials, and frozen in liquid nitrogen. In the case of chinook salmon, however, the livers were frozen over dry ice. The livers were then transferred to the laboratory on dry ice and held at -60°C until analyses for AHH activities were performed. Hematocrits were measured with blood taken from the caudal vein before removal of the liver.

Chemical Analyses of Chlorobiphenyls and Hydrocarbons--

The procedures for hydrocarbon analyses of fish tissues were essentially those reported by Warner (1976). Chlorobiphenyls in diets were analyzed by gas-liquid chromatography (GLC) with electron-capture detection by the procedures of Gruger et al. (1975), and in fish tissues by an adaptation of the Warner method for hydrocarbons. Tissue samples consisted of kidney, liver, and eviscerated, headless, tailless carcass; the latter being essentially muscle tissue, bone, skin, and fat.

The analyses of tissues were performed in cooperation with the NOAA National Analytical Facility (NAF). The modifications developed by NAF for adapting the procedures of Warner (1976) were done in the following respects:

1. Tissue digestions were carried out in alkali, overnight at 30°C and in Teflon-lined screw-capped centrifuge tubes. This manner of digestion minimized losses of volatile compounds.

2. Silica gel chromatography was carried out with 100-200 mesh silica gel (MC/B Manufacturing Chemists No. SX0144-06), which is a coarser, more uniform grade than called for by the Warner procedure (Warner, 1976).

3. High-resolution capillary columns, rather than packed columns, were used in GLC analyses. A 20 m by 0.26 mm WCOT glass capillary coated with SE-30 stationary phase was operated at 2 ml helium per min flow, with temperature programming from 60° to 250°C. Detection was by hydrogen-flame ionization. It was possible to resolve the test compounds, both chlorobiphenyls and the hydrocarbons together, under optimum GLC conditions.

Analyses of Enzyme Activities in Livers--

AHH activity was determined by a modification of the procedure of DePierre et al. (1975). Nonenzymatic background conversions were measured with either boiled microsomes or by using reactions that contained a stop solution before the addition of the substrate. Microsomal protein was determined by an automatic system (Technicon Instruments Corp., Tarrytown, N.Y.) adapted to the method of Lowry et al. (1951). Bovine serum albumin was used as a protein standard.

For optimum specific activity of AHH, reaction mixtures contained the following: 0.8-1.0 mg microsomal protein, 3 mM-$MgCl_2$, 1.1 mM-NADPH, 62.5 mM-tris hydrochloride buffer, and 66 μM-tritiated-benzo[a]pyrene (12.5 mCi/mmole). The final volume was 1 ml. The pH of 7.5 was used in reactions for coho salmon, while pH 7.8 was used initially for chinook salmon

AHH reactions but changed to pH 7.5 for analyses of reactions at the seventh day and thereafter (cf., Results and Discussion). The reaction mixtures were preincubated for 10 min in a shaking water bath at $25 \pm 0.2^{\circ}$C before the initiation of the reactions by the addition of 20 l of acetone containing the benzo[a]pyrene. Duplicate reactions were incubated in 15 x 125 mm open culture tubes in subdued light. After 20 min at 25°C, reactions were stopped; reaction products were extracted and analyzed by the method of DePierre et al. (1975). Radioactivities of 0.3 ml aliquots of neutralized aqueous phase in 15 ml "PCS" (Amersham/Searle Corp., Arlington Heights, Ill.) were measured in a scintillation spectrometer (Packard Tri-Carb model 3003), with corrections determined for background and quenching. AHH activities were calculated as nmoles products/mg microsomal protein/20 min. The Student's t test was applied to AHH activity values to compare differences between control and test fish.

Tritiated benzo[a]pyrene was purified according to DePierre et al. (1975), except the hexane solution of substrate was dried over sodium sulfate prior to making an acetone stock solution. The specific activity of the acetone solution was measured weekly. Purified benzo[a]pyrene was stored at -20°C under nitrogen for up to four weeks.

Results and Discussion

Chemical Analyses--

The analyses for chlorobiphenyls and petroleum hydrocarbons, which were contained in the diets, in liver, kidney, and eviscerated, headless and tailless carcass from the coho salmon revealed that chlorobiphenyls readily accumulated in each tissue after the fish were maintained for 1 to 4 or 5 weeks on those diets containing the chlorobiphenyls (Tables 1, 2, 3). Moreover, no hydrocarbons clearly originating from the mixtures in the diets were detected in liver, kidney, and the eviscerated carcass of fish which were maintained for up to 4 weeks on diets containing the hydrocarbon mixture. Incidentally, n-pentadecane and 1-phenyldodecane were found in all tissues irrespective of whether the diets contained the hydrocarbons. In the analyses, the limit of detection for each chlorobiphenyl and hydrocarbon was found to be 20 ng/g dry weight of tissue.

Radiochemical tracer studies showed that 17% of an administered dose of naphthalene is accumulated as parent hydrocarbon in coho salmon fingerlings, in 14°C freshwater, in 24 hr (Roubal et al., 1977). If hydrocarbons accumulate in 200-g coho salmon, which are maintained in 7°C seawater, to the same degree that naphthalene accumulates in coho fingerlings, which are maintained in 14°C freshwater, then the analyses should have revealed the presence of the hydrocarbons originating from the diets. The apparent absence of the dietary hydrocarbons suggests that those compounds were either poorly absorbed in the stomach and intestines, or readily metabolized to form other products in the body, or readily excreted from the body. In any case, any amount that may have accumulated was below the detection limit.

Reaction parameters for analyses of AHH activities--It was recognized that reaction conditions for determining specific activities of AHH from mammals cannot necessarily be applied directly for fish. Thus, the reaction

TABLE 1. CONCENTRATIONS OF CHLOROBIPHENYLS IN COHO SALMON BODY TISSUES [a]

Diet	Time	BP	2-Cl-BP	2,2'-Cl_2-BP	2,4'-Cl_2-BP	2,5,2'-Cl_3-BP	2,5,2',5'-Cl_4-BP
	Week	- - - - - - - - - - - - - ng/g dry weight of tissue - - - - - - - - - - - - - - - -					
5 ppm Chlorobiphenyls	1	36.1	40.4	107	125	120	80.3
	3	44.0	91.4	175	182	190	104
	5	Tr	37.0	104	146	209	234
	6	ND	240	167	267	345	218
	8	ND	ND	48.7	134	226	414
10 ppm of 50-50 Mixture, Chlorobiphenyls & hydrocarbons	1	28.7	32.8	125	89.5	182	27.9
	2	ND	79.9	173	143	320	203
	3	28	32.0	165	68.6	168	112
	5	Tr	41.0	239	203	525	796
	6	ND	139	172	207	708	736
	8	ND	ND	55.4	54.5	124	66.6

[a] ND = not detected; Tr = trace. Limit of detection was 20 ng/g dry weight. Abbreviations: BP, biphenyl; 2-Cl-BP, 2-chlorobiphenyl; 2,2'-Cl_2-BP, 2,2'-dichlorobiphenyl; 2,4'-Cl_2-BP, 2,4'-dichlorobiphenyl; 2,5,2'-Cl_3-BP, 2,5,2'-trichlorobiphenyl; 2,5,2'5'-Cl_4-BP, 2,5,2'5'-tetrachlorobiphenyl. The body tissue samples consisted of the residual tissues remaining after removal of heads, tails, and viscera.

TABLE 2. CONCENTRATION OF CHLOROBIPHENYLS IN COHO SALMON KIDNEY TISSUES

Diet	Time	BP	2-Cl-BP	2,2'-Cl_2-BP	2,4'-Cl_2-BP	2,5,2'-Cl_3-BP	2,5,2'5'-Cl_4BP
	Week	- - - - - - - - - - - -	- - ng/g dry weight	of tissue - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -
5 ppm Chlorobiphenyls	1	Tr	45.9	61.8	40.2	47.7	20.6
	3	25.0	74.4	59.9	149	36.2	210
	5	ND	ND	37.8	59.2	71.8	Tr
	6	ND	ND	ND	ND	Tr	Tr
	8	ND	ND	ND	Tr	70.9	Tr
10 ppm of 50-50 Mixture, Chlorobiphenyls & hydrocarbons	1	Tr	55.5	102	62.5	112	Tr
	2	ND	ND	Tr	ND	Tr	ND
	3	ND	ND	ND	ND	Tr	ND
	5	ND	ND	71.7	88.1	198	ND
	6	46.8	33.8	86.2	80.6	155	78.4
	8	ND	ND	59.3	51.2	99.9	Tr

[a] ND = not detected; Tr = trace. Limit of detection was 20 ng/g dry wt.
Abbreviations of biphenyls same as for Table 1.

TABLE 3. CONCENTRATION OF CHLOROBIPHENYLS IN COHO SALMON LIVER TISSUES

Diet	Time	BP	2-Cl-BP	2,2'-Cl_2-BP	2,4'-Cl_2-BP	2,5,2'-Cl_3-BP	2,5,2',5'-Cl_4-BP
	Week	- - - - - - - - - - - - - - ng/g dry weight of tissue - - - - - - - - - - - - - -					
5 ppm Chlorobiphenyls	1	41.6	133	80.5	81.6	69.3	ND
	3	37.8	155	108	159	120	Tr
	5	ND	ND	45.6	78.1	92.3	Tr
	6	ND	ND	ND	Tr	ND	ND
	8	ND	ND	33.9	103	146	20.8
10 ppm of 50-50 Mixture, Chlorobiphenyls & hydrocarbons	1	44.1	103	156	101	183	Tr
	2	ND	ND	ND	ND	ND	ND
	3	Tr	ND	219	109	221	Tr
	5	ND	ND	156	141	313	ND
	6	ND	39.7	38.7	102	246	184
	8	ND	ND	68.4	74.8	161	Tr

[a] ND = not detected; Tr = trace. Limit of detection was 20 ng/g dry wt. Abbreviations of biphenyls same as for Table 1.

parameters for measuring activities of hepatic AHH were determined for coho and chinook salmon. Also, it was necessary to determine the conditions for handling and storing tissues prior to their use in assays of AHH activity.

The data in Tables 4 and 5 show that activities of hepatic AHH in coho salmon and rainbow trout (Salmo gairdneri), respectively, are greatest in microsomal fractions. Rainbow trout microsomes served as a reliable reference material for periodic validation of the method for measuring the AHH activity. There was essentially no loss in AHH activity when microsomes were suspended in 0.25 M-sucrose and stored at -60°C for up to 5 months (Table 4). This information was important because samples of suspended microsomes were held frozen for 2-4 months before final analyses. Also, repeated thawing and refreezing of suspended microsomes apparently resulted in a loss of one-third of the AHH activity (compare 3.4 and 2.1 nmoles/mg for 2-day stored salmon parr microsomes, in Table 4); therefore, hepatic AHH assays were performed only with once-frozen microsomes.

Information about the stability of the PAH substrate used in AHH assay reactions was important to the reproducibility of the assay procedure. Thus, the effects of storage and exposure to air of ^{3}H-benzo[a]pyrene in acetone, for up to 65 days, were investigated. The results given in Table 6 show that oxidation products are progressively formed upon repeated intermittent exposure of the substrate to air. In between the exposures, the solutions were held at -20°C. Protection with nitrogen did not entirely prevent oxidation of benzo[a]pyrene in solution. It was important to prepare fresh benzo[a]pyrene substrate after 10-14 days; otherwise, the nonenzymatic analytical blank values became undesirably large.

It was important to establish the optimum concentrations of cofactors, NADPH and magnesium ion, in the AHH reactions. Data for a Lineweaver-Burk calculation from AHH reaction rates, for 0.05 to 5 mM NADPH, are given in Table 7. The concentration of NADPH employed throughout the experiments compares well with a calculated Michaelis constant of 1.33 mM. In the AHH reaction employed in this study, higher specific activities were obtained (Table 8) when fresh preformed NADPH was used, rather than when a NADPH-generating system was employed in situ. In Table 9, NADPH and $MgCl_2$ are shown for comparisons in reactions to indicate the requirements for higher AHH activities. Manganese was without effect, in spite of its use in the method adapted from mammal studies (DePierre et al., 1975).

A study of incubation temperatures of the enzymatic reactions revealed that 25°C is optimum for the salmon hepatic microsomal AHH (Table 10). The optimum temperature generally is lower than for other animals (Adamson, 1967). Optimum reaction times were determined. The data in Table 11 indicate that the AHH reaction rate is linear to 20 min.

The pH optimum for determinations of hepatic microsomal AHH activity for the coho salmon was the same (pH 7.5 in Tris buffer) as with microsomes from mammalian livers (Nebert and Gelboin, 1968; DePierre et al., 1975). By comparison, the same determinations for chinook salmon showed the optimum pH

TABLE 4. TIME EFFECTS OF TISSUE STORAGE AT -60°C ON MICROSOMAL ARYL HYDROCARBON HYDROXYLASE (AHH) ACTIVITY [a]

Fish	Tissue preparation	Time	AHH activity
		Days	nmoles products/mg microsomal protein/ 20 min.
Coho salmon parr	Whole minced liver	14	1.6[b]
	Liver homogenate	14	1.8[b]
	9,000 x g supernate	14	2.7[b]
	microsomes	14	2.3
Coho salmon parr	Microsomes	0	4.9
	Microsomes	1	4.5
	Microsomes	2	3.4
	Microsomes, 3 x thaw-refreeze	2	2.1
Rainbow trout	Microsomes	0	5.1
	Microsomes	3	5.1
	Microsomes	6	4.8
	Liver	6	4.0
	Microsomes	13	4.5
	Microsomes	38	4.7
	Microsomes	79	5.0,5.7
	Microsomes	149	4.6

[a] Incubation mixture contained 3 μmoles MgCl$_2$, 1.1 μmoles NADPH (reduced nicotimamide adenine dinucleotide phosphate), 62.5 μmoles tris hydrochloride, pH 7.5, 66 nmoles ^{3}H-benzo[a]pyrene (12.5 mCi/mmole), and 0.8 mg microsomal protein (from 100,000 g pellet resuspended in 4 ml of 0.25 M-sucrose/g liver), in 1 ml, at 25°C in air.

[b] AHH activity based on proteins of tissue preparation, rather than on microsomal proteins.

TABLE 5. INTRACELLULAR DISTRIBUTION OF AHH ACTIVITY [a]

Cellular fraction	Fish # 969	Fish # 995	Fish #1004	Fish #1008
	- - - nmoles products formed/mg protein/20 min - - -			
Whole homogenate	2.91	2.14	3.66	2.46
9,000 x g pellet	1.70	2.00	5.34	1.96
Microsomes [b]	6.77	4.82	8.67	5.64
Solubles [c]	nil	nil	nil	nil

[a] Preparations from rainbow trout livers. Reaction conditions like those reported in Table 4.

[b] 100,000 x g pelleted material.

[c] 100,000 x g supernatant fraction.

TABLE 6. EFFECTS OF SUBSTRATE STORAGE TIME ON AHH ACTIVITY BLANK VALUES: STORAGE OF A ^{3}H-BENZO[A]PYRENE SOLUTION

Storage time	Blank values [a]
Days	nmole oxidation products
0	0.4
8	0.45
10	0.36
16	0.62
17	0.53
23	0.45
24	0.63
24	0.50
24	0.58
31	0.94
33	0.82
39	0.90
40	1.36
40	1.53
46	0.91[b]
65	2.3

[a] Storage container opened to air on each day measured. Stored at -20°C in between times. Tritium activity, 0.01μCi/nmole.

[b] A sample stored unopened under nitrogen at -20°C for 46 days.

TABLE 7. EFFECTS OF NADPH CONCENTRATION IN BENZO[A]PYRENE HYDROXYLASE REACTIONS OF CHINOOK SALMON HEPATIC MICROSOMES

NADPH	Benzo[a]pyrene hydroxylase activity [a]
µmoles	nmoles products/mg protein/20 min
5	0.823
3	0.816
2	0.792
1	0.686
0.5	0.667
0.05	0.061

[a] Km for NADPH is 1.33 mMolar. Reaction conditions like those reported in Table 4, except at pH 7.8.

TABLE 8. AHH ACTIVITY INFLUENCED BY CONDITIONS OF NADPH IN ENZYME REACTIONS

NADPH-system	Benzo[a]pyrene hydroxylase activity	
	Coho salmon	Rainbow trout
	- - -nmoles products/mg protein/20 min - -	
Generating system [a]	1.63 (88%)	3.36±1.4 (72%)
1.1 µmoles, fresh solution	1.84 (100%)	4.69±0.4 (100%)
1.1 µmoles, 48 hr frozen solution	--	2.91 (58%)

[a] NADPH generated by method of DePierre et al. (1975) using isocitrate and isocitrate dehydrogenase with NADP.

TABLE 9. COFACTOR REQUIREMENTS FOR BENZO[A]PYRENE HYDROXYLASE REACTIONS WITH CHINOOK SALMON LIVER PREPARATIONS

Reaction system	Benzo[a]pyrene hydroxylase activity
	nmoles products formed/mg protein/20 min
Complete[a]	1.38
Minus $MgCl_2$	0.93
Minus NADPH	0.34

[a] Reaction conditions were the same as reported in Table 4, except at pH 7.74.

TABLE 10. BENZO[A]PYRENE HYDROXYLASE ACTIVITY OF COHO SALMON HEPATIC MICROSOMES AT VARIOUS TEMPERATURES OF THE ENZYME ANALYSIS REACTION [a]

Temperature	Benzo[a]pyrene hydroxylase activity
°C	nmoles products formed/mg protein/20 min
10	1.29
15	2.14
20	2.29
25	2.23
40	0.63

[a] Reaction conditions were the same as reported in Table 4.

TABLE 11. AHH ACTIVITY OF COHO SALMON HEPATIC MICROSOMES AS A FUNCTION OF TIME OF ENZYME ANALYSIS REACTION [a]

Time	AHH activity
min	nmoles products/mg protein
2	0.27
5	0.56
8	0.90
12	2.1
16	2.5
20	3.9
25	3.3
30	3.6
57	5.2
74	6.1

[a] Reaction conditions were the same as reported in Table 4.

to be 7.8; however, following exposures of chinook to petroleum hydrocarbons the optimum appeared to shift to pH 7.5 (cf., Table 12). This shift, which occurred after one week, was examined only at the 5 ppm concentration of test compounds. A shift of pH 7.8 to pH 7.5 can result in significantly different AHH activities. The pH shift was not observed for the coho salmon.

Observed activities of liver microsomal AHH in relation to test compounds--The specific activities of AHH, as benzo[a]pyrene hydroxylase, found for various groups of fish are presented in Table 13 (coho) and Table 14 (chinook).

Control coho salmon (Table 13) exhibited gradual increases in mean specific activities of AHH during the first four weeks of feeding. However, only the maximum specific activity at the fourth week and the minimum specific activities at the sixth and seventh weeks are significantly different ($P<0.05$) from each other; activities at other times were statistically the same as for the controls. These differences from maximum to minimum may be associated with the condition of the fish at later experimental periods, as will be discussed below in the section on condition of fish.

The coho fed 1 ppm chlorobiphenyls exhibited no significant differences in AHH activities compared to controls. At the 5 ppm concentration in food, the chlorobiphenyls produced a significant ($P<0.05$) decrease in AHH activities after three weeks, but thereafter there were no differences compared to controls.

TABLE 12. EXPOSURES TO CHLOROBIPHENYLS AND HYDROCARBONS: INFLUENCE ON OPTIMUM pH OF AHH ACTIVITIES FOR CHINOOK SALMON HEPATIC MICROSOMES

Treatment [a]	AHH activity	Observed pH[b]	Optimum pH
	nmoles products/ mg protein/20 min		
Control, #1 zero time	0.44	7.29	7.9
	0.48	7.75	
	0.66	7.81	
	0.70	7.96	
	0.56	8.42	
Control, #2 zero time	1.02	7.32	7.8
	1.38	7.74	
	1.75	7.82	
	1.44	8.01	
5 ppm chlorobiphenyls, 3 days	0.44 ± 0.24 (n=4)	7.5	7.8
	0.64 ± 0.25 (n=6)	7.8	
5 ppm chlorobiphenyls, 7 days	0.43	7.23	7.8
	0.46	7.43	
	0.56	7.76	
	0.26	7.96	
5 ppm chlorobiphenyls, 14 days	0.30 ± 0.18 (n=5)	7.5	7.8
	0.43 ± 0.34 (n=5)	7.8	
5 ppm hydrocarbons, 3 days	0.18 ± 0.17 (n=6)	7.5	7.8
	0.54 ± 0.29 (n=5)	7.8	
5 ppm hydrocarbons, 7 days	0.25	7.27	7.5
	0.26	7.50	
	0.07	7.84	
	0.06	8.05	
	0.02	8.53	
5 ppm hydrocarbons, 14 days	0.42 ± 0.22 (n=10)	7.5	7.5
	0.30 ± 0.19 (n=10)	7.8	
10 ppb mixed chlorobiphenyls and hydrocarbons, 14 days	0.78 ± 0.42 (n=5)	7.5	7.8
	0.91 ± 0.54 (n=5)	7.8	

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[a] Chinook salmon fed various diets and sampled at 0, 3, 7, and 14 days, as described in text.

[b] pH measurements made with a research pH meter (Radiometer-Copenhagen, Type PHM-25, London Co., Westlake, Ohio).

TABLE 13. SPECIFIC ACTIVITIES OF HEPATIC MICROSOMAL ARYL HYDROCARBON HYDROXYLASE (AHH) IN COHO SALMON FED A CONTROL DIET AND DIETS CONTAINING TEST COMPOUNDS [a]

Time, week [b]	Control	Chlorobiphenyls		Hydrocarbons		Mixed chlorobiphenyls and hydrocarbons	
		1 ppm	5 ppm	1 ppm	5 ppm	2 ppm	10 ppm
1	0.58 ± 0.35	0.52 ± 0.29	0.90 ± 0.54	0.79 ± 0.48	0.60 ± 0.41	0.73 ± 0.42	1.09 ± 0.25[c]
2	0.61 ± 0.32	0.72 ± 0.29	0.74 ± 0.19	0.70 ± 0.55	1.09 ± 0.69	1.06 ± 0.45[d]	0.73 ± 0.26
3	0.72 ± 0.31	0.76 ± 0.37	0.43 ± 0.29[d]	0.80 ± 0.50	0.71 ± 0.30	0.81 ± 0.54	0.61 ± 0.41
4	0.89 ± 0.59	0.68 ± 0.41	0.59 ± 0.24	0.63 ± 0.33	0.88 ± 0.46	0.72 ± 0.56	0.70 ± 0.56
5	0.56 ± 0.25	0.70 ± 0.50	0.51 ± 0.36	0.32 ± 0.23	0.60 ± 0.34	0.74 ± 0.36	0.71 ± 0.43
6	0.37 ± 0.18	0.32 ± 0.27	0.36 ± 0.34	0.22 ± 0.13	0.33 ± 0.18	0.42 ± 0.22	0.45 ± 0.25
7	0.37 ± 0.14	--	0.49 ± 0.30	--	0.69 ± 0.46	0.54 ± 0.29	0.47 ± 0.33
8	0.44 ± 0.24	0.38 ± 0.21	0.48 ± 0.13	0.44 ± 0.46	0.57 ± 0.23	0.27 ± 0.19	0.39 ± 0.26

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[a] Ten fish per group per time period. Mean ± S.D.; AHH activity units as nmoles of benzo[a]pyrene hydroxylated products per mg of microsomal proteins per 20 minutes.

[b] Weeks 1 - 4 for feeding chlorobiphenyls and hydrocarbons; weeks 5 - 8 for depurations by feeding control diet to all groups.

[c] Statistically different from controls ($P \leq 0.01$)

[d] Statistically different from controls ($P \leq 0.05$)

TABLE 14. SPECIFIC ACTIVITIES OF HEPATIC MICROSOMAL ARYL HYDROCARBON HYDROXYLASE (AHH) IN CHINOOK SALMON FED A CONTROL DIET AND DIETS CONTAINING TEST COMPOUNDS

Time	AHH activity[a]			
	Control	5 ppm Chlorobiphenyls	5 ppm Hydrocarbons	10 ppm Chlorobiphenyls and Hydrocarbons
Day				
0	1.09 ± 0.45[b]	-	-	-
0	0.96 ± 0.56	-	-	-
1	(1.22 ± 0.55)[c]	0.76 ± 0.24[d]	0.82 ± 0.4	0.67 ± 0.23[d]
2	(1.22 ± 0.55)[c]	0.96 ± 0.22	0.77 ± 0.35[d]	0.85 ± 0.38
3	(1.22 ± 0.55)[c]	0.72 ± 0.37[d]	0.86 ± 0.48	0.62 ± 0.47[e]
4	(1.22 ± 0.55)[c]	0.44 ± 0.28[f]	0.50 ± 0.27[e]	0.91 ± 0.48
7	1.39 ± 0.63	0.83 ± 0.49[d]	0.63 ± 0.36[e]	0.52 ± 0.34[e]
14	1.08 ± 0.25	0.96 ± 0.45	0.65 ± 0.32[e]	1.27 ± 0.66
21	1.18 ± 0.56	0.60 ± 0.35[d]	0.73 ± 0.42[d]	0.65 ± 0.45[d]

[a] Ten fish per group AHH activity (mean ± S.D.) as nmoles products formed /mg microsomal protein/20 min.

[b] Activity related to fish before the standard exposures to MS-222

[c] Average value of controls at 0 and 7 days

[d] Significantly different from controls ($P \leq 0.05$)

[e] Significantly different from controls ($P \leq 0.01$)

[f] Significantly different from controls ($P \leq 0.001$)

The coho fed 1 ppm hydrocarbons alone exhibited no increase in the AHH activity. In addition, the 5 ppm hydrocarbon feeding resulted in no significant increases in AHH activity in coho hepatic microsomes (Table 13). In Table 13, the data indicate that fish fed the mixtures of chlorobiphenyls and hydrocarbons together, i.e., 50-50 in 2 ppm and 10 ppm concentrations, respectively, exhibited AHH induction in one to two weeks. Those increases in AHH activities were significant ($P<0.01$ and $P<0.05$). No other significant differences were found at other times for the fish fed mixtures of chlorobiphenyls and hydrocarbons.

Inasmuch as the results with the test compounds in coho salmon suggested AHH induction within one to two weeks, the experiment with chinook salmon included analyses of AHH activities more frequently in the first week of exposures in an effort to determine more precisely the time that significant change occurs in AHH activity. Only the effect of a 5 ppm concentration of test compounds was studied (Table 14), because that concentration caused the most significant change (increase) in AHH activity in coho in the shortest time.

The control chinook salmon exhibited no significant change in hepatic AHH activity in three weeks of feeding on the basal diet. Table 14 includes data for chinook salmon before and after exposure to the anesthetic MS-222. The data indicate no effect of anesthesia on the hepatic AHH activity. The anesthetic was necessary for weighing and sorting fish, so its possible effect on the enzymatic system was important.

Examination of the data in Table 14 reveals significant decreases in chinook hepatic AHH activities in relation to the three examined test diets. Significant differences compared to controls were generally consistent, but varied in degree of confidence ($P<0.001$ to $P<0.05$). No induction of hepatic AHH activity was observed in the chinook salmon.

Conditions of Coho Salmon--

An understanding of the general health of experimental animals was considered to be important; therefore, the fish were monitored for visible signs of pathological abnormalities. By the fourth week of exposures to the dietary test compounds, it appeared that many coho salmon were developing health problems.

Some mortalities were observed throughout the experiments, but these could not be attributed to the composition of the diets. At the beginning of the study, all fish appeared healthy. However, the test coho salmon exhibited random and progressive signs of fin lesions (fin rot disease), kidney lesions, enlarged spleens, and orange pigmentations of livers during the course of the study. Moreover, the control salmon appeared generally healthier than the salmon in the test groups. At the final eighth week sampling, all fish exhibited the abnormal health signs. Hematocrits of coho salmon from control and test groups, measured during the fourth and fifth weeks, ranged from 4% to 50%, with mean ±S.D. values of 34 ± 12 and 32 ± 14, respectively.

On the whole, average values of hematocrits appear to be poor indicators of the presence of pathology; however, individual hematocrits of 20% and less may be indicative of a forthcoming disease condition in the fish. More hematocrit information relating to healthy salmon is needed.

The body weight of fish can be useful in judging effects of diet and test compounds on fish. Table 15 presents the weight data for coho salmon used for AHH analyses: No correlation is evident between diets and weight. No overall growth occurred among controls or test coho salmon, which was consistent with the feeding plan.

A recalculation of hepatic AHH activities for coho salmon based on visible signs of good health versus pathological symptoms did not change the results and interpretations discussed above for the effects of test compounds on the enzyme system.

Conditions of Chinook Salmon--

The data is Table 16 presents the body weights of chinook salmon taken for AHH analyses. Comparisons of mean weights over a 49-day period indicate slow growth rates for all four groups, which is according to the feeding schedule and does not reflect obvious differential effects of xenocompounds.

The incidence of physical signs of lesions found in the chinook salmon used for analysis of AHH activities are summarized in Table 17. All fish were in excellent condition at the start and no signs of pathology were evident until after one week of feeding the test compounds. It was not until four weeks of exposures to the test compounds that kidney lesions were observed. In general, the control chinook salmon were healthier than the test fish.

Measurements of hematocrits were made for the chinook salmon. These measurements were for all chinook salmon taken for the analyses of AHH activity, because of our concern for the condition of the fish after the experience with the coho salmon. The values for the hematocrits are presented in Table 18. Hematocrits ranged randomly from 25±11% to 40±10% with no apparent relationship to pathology.

Many factors affect hematocrits or blood cell counts in fish, such as water temperature, state of maturity, spawning cycle, metabolic activity, and sampling techniques (Katz, 1949; Barnhart, 1969). If we assume that values of 32% to 52% found by Wedemeyer and Chatterton (1971) for juvenile hatchery coho salmon (10-15 g size) are reasonable values for mature salmon in seawater, then perhaps salmon with hematocrits of about 30% may be regarded as bordering on developing a health problem.

Interpretation of Results--

The highest activities of hepatic AHH were observed when the hydrocarbons together with the chlorobiphenyls were fed to the coho salmon. Results suggest that the concentration of these xenobiotics influenced the induction of AHH in coho salmon; i.e., the induction occurred one week later when the fish were fed 2 ppm of the 50-50 mixture of chlorobiphenyls and hydrocarbons than when the fish were fed 10 ppm of the mixtures of compounds.

Chlorobiphenyls alone did not cause increased AHH activity in coho salmon; however, the results suggest that these compounds did influence the hepatic AHH enzyme system when administered together with hydrocarbons. The data showed that salmon treated with 10 ppm of the 50-50 mixture exhibited higher AHH activity after one week of feeding than did the controls. Fish that were fed only 5 ppm of hydrocarbons or 5 ppm of chlorobiphenyls, for one week, exhibited no change in AHH activity. In addition, induction of AHH activity occurred when the fish were fed 2 ppm of the 50-50 mixture for two weeks, but not when either chlorobiphenyls or hydrocarbons were administered

TABLE 15. BODY WEIGHTS OF COHO SALMON TAKEN FOR AHH ANALYSES [a]

Time	Control	Chlorobiphenyls		Hydrocarbons		Mixed chlorobiphenyls and hydrocarbons	
		1 ppm	5 ppm	1 ppm	5 ppm	2 ppm	10 ppm
Week	- Grams -						
1	222 ± 41	196 ± 64	218 ± 37	198 ± 56	225 ± 24	215 ± 40	242 ± 38
2	222 ± 56	216 ± 44	210 ± 49	176 ± 38	211 ± 32	238 ± 50	204 ± 36
3	194 ± 38	230 ± 34	184 ± 32	200 ± 23	182 ± 42	208 ± 31	181 ± 39
4	196 ± 31	220 ± 47	210 ± 45	209 ± 51	213 ± 43	224 ± 38	180 ± 36
5	199 ± 36	253 ± 19	189 ± 33	213 ± 41	210 ± 41	209 ± 41	207 ± 46
6	199 ± 38	198 ± 51	210 ± 37	191 ± 39	172 ± 30	170 ± 34	208 ± 44
7	172 ± 34	--	227 ± 33	--	224 ± 26	194 ± 54	210 ± 45
8	209 ± 21	178 ± 37	213 ± 56	185 ± 84	236 ± 48	212 ± 39	203 ± 53

[a] Ten fish per group for mean weights ± standard deviations.

Table 16. BODY WEIGHTS OF CHINOOK SALMON TAKEN FOR AHH ANALYSES [a]

Time	Control	5 ppm Chlorobiphenyls	5 ppm Hydrocarbons	10 ppm mixed Chlorobiphenyls and hydrocarbons
Day	- - - - - - - - - - - - - - - Grams	- - - - - - - - - - - - - - - - -		
0	75 ± 15	-	-	-
1	-	82 ± 17	79 ± 18	76 ± 16
2	-	66 ± 10	73 ± 15	70 ± 13
3	-	81 ± 18	74 ± 10	82 ± 14
4	-	81 ± 18	76 ± 16	82 ± 14
7	72 ± 17	90 ± 18	83 ± 14	89 ± 15
14	86 ± 18	71 ± 8	79 ± 15	89 ± 19
21	75 ± 11	104 ± 39	79 ± 15	78 ± 8
28	95 ± 19	102 ± 21	91 ± 13	108 ± 25
31	86 ± 15	109 ± 17	90 ± 18	97 ± 14
36	88 ± 13	92 ± 11	97 ± 17	103 ± 16
42	96 ± 17	100 ± 39	-	105 ± 15
49	99 ± 19[b]	98 ± 27	116 ± 24[c]	100 ± 23[c]

[a] Ten fish per group for mean weights ± standard deviations, except as noted specifically.

[b] n = 59 for mean ± S.D.

[c] n = 19 for mean ± S.D.

TABLE 17. PERCENTAGE INCIDENCE OF FIN ROT DISEASE OBSERVED FOR CHINOOK SALMON TAKEN FOR AHH ANALYSES [a]

Time	Control	5 ppm chlorobiphenyl	5 ppm hydrocarbons	10 ppm mixed chlorobiphenyls and hydrocarbons
Day	Percent			
0	0	0	0	0
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
7	0	50	30	40
14	10	50	30	80
21	40	90	40	80
28	20	70 (20)	70 (30)	70
31	10 (20)	100 (20)	50 (20)	80 (10)
36	20 (10)	70 (20)	20 (20)	50 (30)
42	20 (10)	20	-	10 (10)
49	10	20 (10)	20 (30)	40 (10)

[a] Ten fish per group. Numbers in parentheses are percentage incidence of observed kidney disease. No kidney disease observed before 28 days.

alone at 1 ppm in the diets for the same time. These findings imply that chlorobiphenyls and aromatic hydrocarbons act together synergistically in inducing hepatic AHH activity in coho salmon.

Results with chinook salmon showed that their hepatic AHH system responded negatively to the test compounds. Perhaps a possible cause for a lowered AHH activity for the chinook salmon enzyme system is linked to the fact that the fish were in seawater in the summer, when temperatures of the water rose to about 13°C. This factor together with other seasonal parameters may have altered xenobiotic metabolism and the AHH activities. Additional research is required to answer questions about

TABLE 18. HEMATOCRITS FOR CHINOOK SALMON TAKEN FOR AHH ANALYSES [a]

Time	Control	5 ppm Chlorobiphenyls	5 ppm Hydrocarbons	10 ppm mixed Chlorobiphenyls and hydrocarbons
Day	- - - - - - - - -Percent packed-cell volume - - - - - - - - - - - -			
1	-	25 ± 5	31 ± 6	26 ± 8
2	-	33 ± 7	35 ± 10	29 ± 4
3	-	30 ± 4	35 ± 4	33 ± 10
4	-	29 ± 5	24 ± 5	30 ± 6
7	33 ± 6	31 ± 5	33 ± 5	34 ± 4
14	37 ± 3	34 ± 7	-	40 ± 2
21	35 ± 7	39 ± 7	38 ± 8	37 ± 10
28	41 ± 3	31 ± 16	32 ± 14	41 ± 4
31	40 ± 9	36 ± 13	39 ± 4	38 ± 11
36	39 ± 11	40 ± 5	40 ± 10	38 ± 10
42	37 ± 9	37 ± 8	-	25 ± 11
49	37 ± 5	33 ± 9	32 ± 12	29 ± 10

[a] Ten fish per group for mean values ± standard deviations.

the effects of chlorobiphenyls and petroleum hydrocarbons together in chinook salmon in the winter or spring, when seawater temperatures are near to $7^{o}C$ or less.

At this point it is premature to suggest what effect a decreased hepatic AHH activity has in chinook salmon exposed to the test xenobiotics; however, it is reasonable to conclude that lower activity of the AHH system may result in decreased metabolism of xenobiotics such as petroleum PAH's. The possibility of decreased metabolism of PAH's may affect the toxicity of these compounds in the species.

A brief statement of findings with other species will illustrate additional complexities of the AHH system. For instance, many xenobiotics may not induce AHH activity in animals or, if so, the extent to which aromatic hydroxylation occurs with different compounds varies considerably among species, as work by Williams (1971) has shown. Benzo[a]pyrene hydroxylase is not especially responsive to many inducers of drug-metabolizing enzymes, including PCB's and certain chlorine-containing pesticides (Fouts, 1973). Furthermore, some compounds exert an opposite effect of inducers. For example, Ahokas et al. (1976) found that pike exposed to domestic and industrial wastes exhibited lower AHH activity than controls.

Our results are consistent with others which show that activities of xenobiotic metabolizing enzyme systems are often lower in fish compared to those in higher animals (Adamson, 1967; Buhler and Rasmusson, 1968). Moreover, while the microsomal xenobiotic metabolizing enzyme systems of fish appear to be similar in many respects to that of mammals (Stanton and Khan, 1975), there are species differences such as the lack of mixed function oxidase induction by phenobarbital in marine fishes (Philpot et al., 1976). Thus, there is no reason to assume that the effects of xenobiotics on hepatic AHH activity for fish will parallel changes brought about by these compounds in mammals. The nature and extent of induction of AHH activity within a given species of fish can also vary among different strains and is related to the geographic origin of the fish (Pedersen et al., 1976). Furthermore, induction of AHH in fish appears to be inversely affected by both water temperature (Dewaide, 1970) and salinity (Gruger et al., 1977). These, and perhaps other factors, make it difficult to make absolute comparisons of results of experiments on the AHH systems in fish between laboratories.

Summary

Coho salmon and chinook salmon were exposed to two mixtures of pure chlorobiphenyls and petroleum hydrocarbons, separately and together, at 1 ppm and 5 ppm of each mixture in Oregon moist pellets. Chemical analyses of coho salmon tissues indicated that the chlorobiphenyls accumulated in the liver, kidney, and residual tissues composed of eviscerated, headless and tailless carcasses; although, hydrocarbons were not detected in these organs and tissues.

Aryl hydrocarbon (benzo[a]pyrene) hydroxylase activities of liver microsomes were compared among test and control salmon of both species in order to determine whether alterations in enzyme activities occurred in relation to the exposures. Optimal reaction conditions were established for measurements of aryl hydrocarbon hydroxylase activities in salmon hepatic microsomes; the conditions indicated temperature differences for reactions of salmon microsomes compared to mammalian microsomes and differences in pH for the reactions of microsomes from coho salmon compared to those from chinook salmon.

Induction of the aryl hydroxylase enzyme occurred in coho salmon, maintained in 7°C seawater, within two weeks of exposure to the hydrocarbons; the induction was also potentiated by the presence of the chlorobiphenyls, which by themselves had no effect on the enzyme activity. A synergistic effect was indicated for the test compounds on the induction of aryl hydrocarbon hydroxylase in coho salmon liver. In chinook salmon maintained in 13°C seawater, the activity of aryl hydrocarbon hydroxylase in hepatic microsomes was depressed by the chlorobiphenyls and hydrocarbons administered singly or together. No interaction of these two classes of xenobiotics on the hydroxylase activity was found with chinook salmon.

THE EFFECTS OF PETROLEUM HYDROCARBONS AND CHLORINATED BIPHENYLS ON THE MORPHOLOGY OF TISSUES OF CHINOOK SALMON

Methods

Samples of skin, gills, intestine, liver, and kidney were taken from three chinook salmon randomly selected from each test group, described in the previous (above) experiments, and the control group at 14, 28, and 49 days (Hawkes et al., 1980). The tissues were fixed in 0.75% glutaraldehyde, 3% formalin, 0.5% acrolein in 0.1 M sodium cacodylate buffer (pH 7.4) with 0.02% $CaCl_2 \cdot H_2O$, and 5.5% sucrose. After a buffer wash (0.1 M sodium cacodylate, 0.02% $CaCl_2 \cdot H_2O$, 5.5% sucrose), specimens were postfixed in 1% osmium tetroxide in buffer. Dehydration with ethanol and embedding in Spurr medium (Spurr, 1969) completed the preparation of the tissues for sectioning with either glass or diamond knives. Sections were prepared with Richardson's stain for light microscopy, and thin sections were triple stained with lead citrate, uranyl acetate, and lead citrate for electron microscopy.

Several staining techniques were used to identify the subcellular inclusions in the intestinal mucosa. For glycoproteins, a modification of the periodic acid-Schiff (PAS) technique for use with plastic-embedded tissue was employed (Nevalainen et al., 1972), and for lipid, Sudan black and Aparicio's stain (Aparicio and Marsden, 1969) were used.

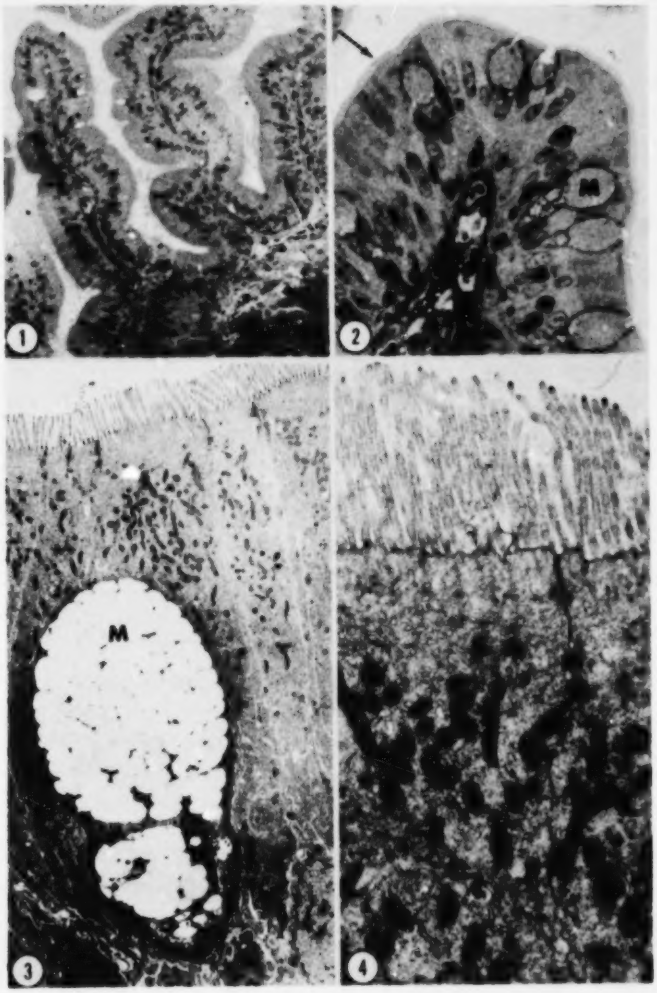
Results

Morphological changes that deviated from the normal appearance of the controls were observed only in liver and intestine. In some of the salmon exposed to chlorinated biphenyls, the rough endoplasmic reticulum was vesiculated and there were degenerating membranes in hepatocytes throughout these liver sections (Hawkes, 1980).

Examination of the intestinal mucosa from 32 of the 36 fish was completed. In samples from four fish the sections passed below the mucosa and were not included in the study. There were minor variations among individuals, but there were uniform patterns of normal morphology among fish of the control group and consistent alterations among the exposed animals. These findings were similar after both the 14- and the 28-day exposures with no apparent increased damage in the 28-day-exposed group. In addition, structural changes in exposed animals persisted at the same level of severity after 21 days of depuration.

Control Group--

The general organization and histology of the intestine of chinook salmon have been described by Greene (1913) and will not be repeated here. Because structural changes occurred in the mucosa of contaminant-fed fish, the normal ultrastructure of that area of the intestine is briefly described (Figs. 1-4). The mucosa contains mucous cells, cylindrical epithelial cells, rodlet cells, and leukocytic cells similar to those reported in rainbow trout (Yamamoto, 1966). Mucous cells in several stages of maturation are present: the most immature are located near the basal regions of the mucosa, and the more mature cells are near the surface and are filled with typical mucin inclusions



FIGURES 1-4. Micrographs of intestine from control chinook salmon. Fig. 1. Light micrograph of intestinal villi. 30 x. Fig. 2. Tip of villus with normal mucous cells (m) and brush border (arrows). 1100 x. Fig. 3. Electron micrograph of columnar epithelial cells and mucous cell (m). 3500 x. Fig. 4. Microvillar surface and upper fourth of a typical columnar cell. Note the aggregation of mitochondria and the few profiles of endoplasmic reticulum. 14,300 x.

sequestered in discrete membrane-bound vesicles. The columnar epithelial cells are stratified and have aggregations of mitochondria near the luminal surface and the nucleus is in the basal region. Granular endoplasmic reticulum, mitochondria, and agranular cytoplasm occupy the midportion of these cells. Multivesiculate bodies and other vesicles are present in the mid- or luminal regions of some of the mucosal cells.

Petroleum-Exposed Group--

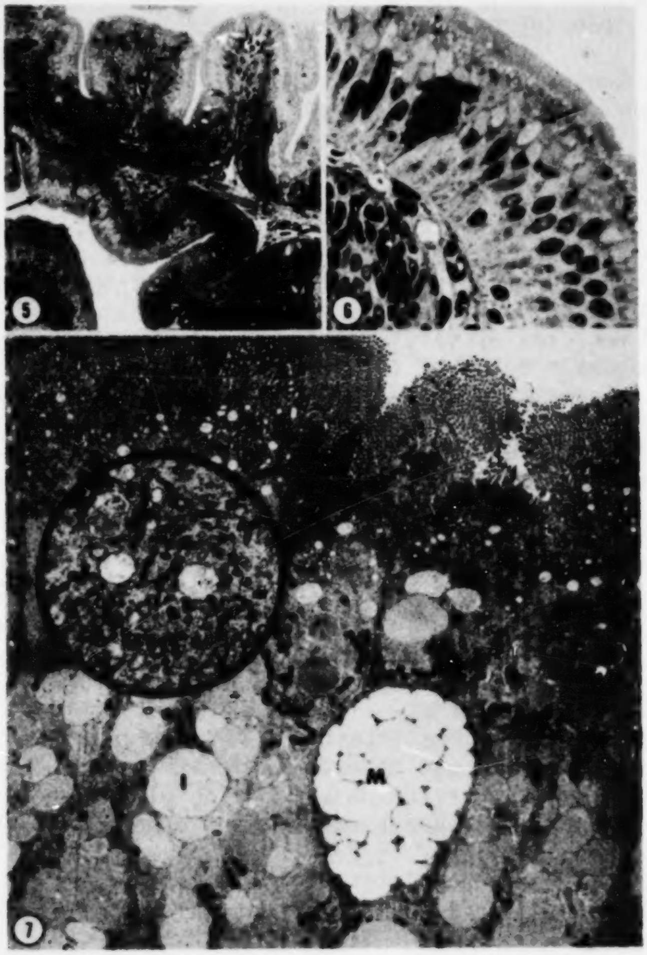
Chinook salmon exposed to the mixture of petroleum hydrocarbons maintained overall integrity of the intestinal mucosa and underlying regions. There was little exfoliation of cells and little focal necrosis (Figs. 5, 6); however, varying degrees of subcellular changes occurred in eight of nine fish examined. Three aberrations were noted: (1) presence of unusual cellular inclusions in the columnar cells of the mucosa, (2) vesiculation of cytoplasm near the luminal surface of columnar cells, and (3) cytoplasmic changes in basal cells of the mucosa. A flocculent or finely granular material, enclosed in membrane-bound vesicles of varying size and electron density, filled the luminal half of columnar cells of the intestine of some fish (Figs. 6, 7). In other individuals, normal-appearing and vesicle-packed cells were interspersed. In all the petroleum-exposed fish, typical goblet cells were present throughout the affected regions. Immediately below the luminal surface of the mucosa, there was a 13-μm zone that contained small vesicles with the flocculent inclusions. The zone also contained profiles of agranular endoplasmic reticulum not observed elsewhere in the cell (Fig. 7).

In tissue from animals with the greatest amount of vesiculation, the basal mucosal cells had an unusually electron-transparent cytoplasm and an increase in granular endoplasmic reticulum.

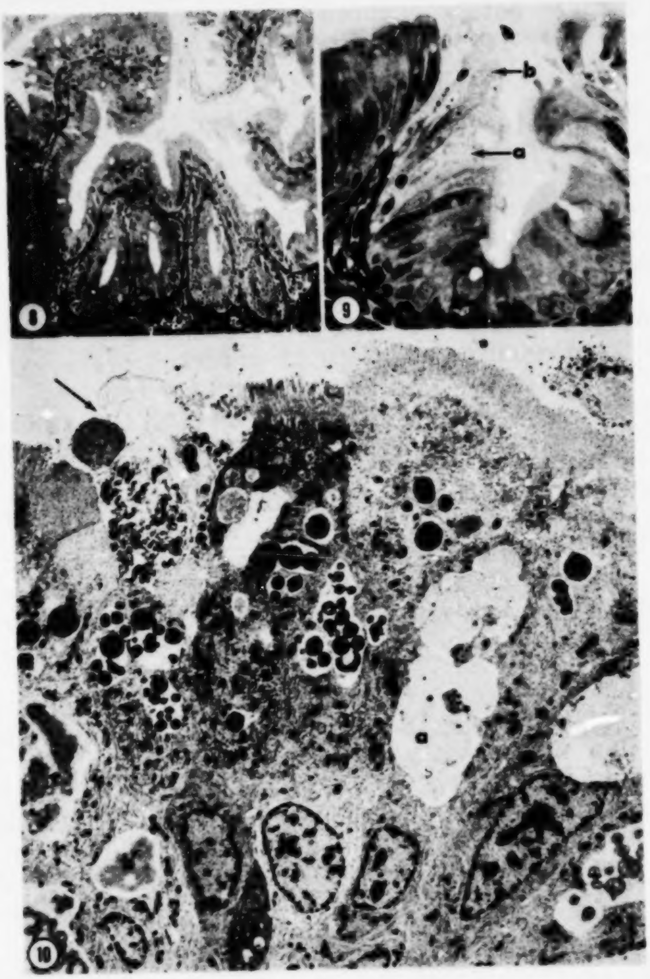
Chlorinated Biphenyl-Exposed Group--

Although the majority of the intestinal cells were intact, there was a noticeable increase in the amount of exfoliation (Figs. 8,9) of the mucosa of the chlorinated biphenyl-treated chinook compared with the control or the petroleum-treated groups. The brush border was intact over most of the mucosa, but was reduced or absent in some areas, even though the cells showed no signs of impending lysis and sloughing. Clusters of columnar cells with abnormal cytoplasmic inclusions were interspersed among normal-appearing cells.

Inclusions in the mucosal cells could be grouped into at least two and possibly three different structural types (Fig. 10). The first consisted of irregularly shaped and variably sized vesicles of relatively electron-transparent material of a finely fibrillar consistency. It was difficult to determine if a limiting membrane was present on the large masses of this material, even though small vesicles were clearly membrane bound. Membranes of nearby mitochondria and granular endoplasmic reticulum were well defined and appeared normal. The second type of inclusion was much more electron dense; the small vesicles were obviously membrane bound. Large aggregates of this material frequently included small foci of electron-transparent material. A possible third type of inclusion was intermediate in density and resembled the second type of vesicle in having small areas of quite clear material.



FIGURES 5-7. Micrographs of intestine from chinook salmon fed a model mixture of 5 ppm petroleum hydrocarbons for 28 days. Fig. 5. Light micrograph of intestinal villi with numerous inclusions (arrow). 30 x. Fig. 6. Higher magnification of columnar cells of the mucosa. The mucous cells (m) appear to be normal. The inclusions (arrow) are restricted to the upper third of the columnar cells. 1100 x. Fig. 7. Electron micrograph of upper third of the portion of the intestinal mucosa. Mucous cell (m), inclusion (i). 5300 x. The insert, a high magnification of the region below the microvillar terminal web, has an abundant network of agranular endoplasmic reticulum. 17,000 x.



FIGURES 8-10. Micrographs of intestine from chinook salmon fed 5 ppm chlorinated biphenyl mixture for 28 days. Fig. 8. Light micrograph of the intestinal mucosa with exfoliation (arrow). 30 x. Fig. 9. Two types of inclusions (a,b) are evident in this light micrograph. 1100 x. Fig. 10. Electron micrograph of the upper portion of mucosal cells with damaged brush border and exfoliation (arrow). The inclusions correspond to the (a) and (b) types in Fig. 9. The finely granular material of the type (a) granule may coalesce to form type (b) with (b') being an intermediate. 3500 x.

Near the luminal surface of these intestinal mucosal cells, there were clusters of small vesicles containing all of the above types of inclusions as well as numerous profiles of agranular endoplasmic reticulum. Nuclei maintained the typical basal location but the mitochondria were distributed throughout the cell. Normal-appearing mucous and rodlet cells were commonly observed.

Petroleum- and Chlorinated Biphenyl-Exposed Group--

The amount of exfoliation of the surface mucosal cells of intestine appeared to increase in seven of eight combined-contaminant-treated fish (Figs. 11,12) compared to the other groups. The brush border was reduced in areas where exfoliation had not occurred, and this reduction may have been a harbinger of impending cell loss.

Cellular changes ranged from slight to pronounced in areas where the mucosa was intact. The columnar shape of affected cells was maintained as was the basic polarity of the cell (i.e., the nucleus remained in the basal region). Mitochondria, however, were interspersed throughout the cytoplasm rather than being clustered near the luminal surface as in the controls. The cytoplasm exhibited several major changes, including alterations in the vesiculation near the brush border, presence of inclusions throughout the cell, an increase in endoplasmic reticulum, and reduction of cytoplasmic density (Fig. 13). A region of agranular endoplasmic reticulum near the luminal surface extended deeply into the cell (Fig. 14). The surface microvilli had deep intertwining extensions in the upper quarter of this region.

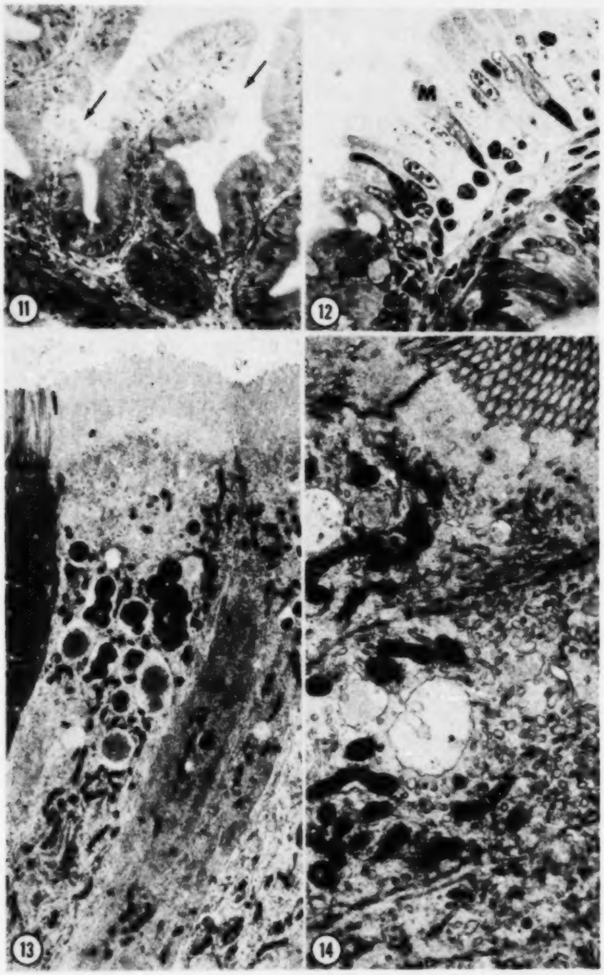
Membrane-bound vesicles of varying size and electron density occurred throughout the cells, but were located primarily in the luminal half of the columnar cells. The inclusions were membrane-bound vesicles of widely ranging sizes and electron densities and had a finely fibrillar structure. The cytoplasmic ground substance frequently appeared much less electron dense, lacked free ribosomes, and had increased profiles of granular endoplasmic reticulum. Columnar cells with these modifications were located near or adjacent to normal-appearing mucuous, rodlet, and columnar cells.

Histochemistry--

Inclusions in the intestinal mucous cells of both normal and contaminant-exposed intestine showed a positive PAS reaction; however, cells with inclusions from all of the contaminant-exposed animals were clearly PAS negative. The inclusions did not stain with Sudan black. Aparicio's staining technique was used to delineate the red mucin from blue cytoplasm of columnar cells and from greenish lipid droplets. The inclusions stained a bluish hue with this technique.

Discussion

Structural changes in the intestine of salmon exposed to the chlorinated biphenyls and petroleum hydrocarbons used in this study involved marked subcellular alterations in intact cells compared with cells of controls (Hawkes et al., 1980). Exfoliation of the intestinal epithelium occurred when the fish were exposed to chlorinated biphenyls or to the mixture of petroleum



FIGURES 11-14. Micrographs of intestine from chinook salmon fed 5ppm each of a petroleum hydrocarbon mixture and a chlorinated biphenyl mixture. FIG. 11. Light micrograph of intestinal villus with several sites of exfoliation (arrows). 30 x. FIG. 12. Mucous cells (m) and columnar cells in a region of sloughing. 1100 x. FIG. 13. Electron micrograph of columnar cell with inclusions from an area of the mucosa in which there was no evidence of exfoliation. 4800 x. FIG. 14. Higher magnification of the base of the microvilli, terminal web, and subadjacent region. Note the abundance of agranular endoplasmic reticulum. 13,000 x.

and chlorinated biphenyls; the combined effect of the two contaminants on epithelial exfoliation appeared much greater than with chlorobiphenyls alone, suggesting an exacerbated interaction. A very clear case of interactive effects was reported by Itokawa et al. (1975) on the effects of polychlorinated biphenyls and alkylbenzene sulfonic acid on rats. Testicular damage occurred only when both contaminants were administered. The rats also showed liver pathology with exposure to the individual contaminants and an increased pathology with combined exposure; this increase appeared to be an additive effect. Therefore, in the same animal and within the same time frame, one tissue showed a clearly enhanced interactive effect from two contaminants whereas another tissue showed an additive change.

The presence of inclusions in the chinook salmon intestine represents distinct changes from the normal columnar cell morphology. We do not know whether the substances were incorporated into the cell from material in the intestinal lumen, were produced by the cell, or can be solely attributed to degenerative changes. The appearance of the inclusions suggests that they were not composed of absorbed food material. Moreover, some of the cells were observed in the process of releasing the material into the lumen of the gut. Histochemical tests clearly demonstrated that the inclusions were not stained by either the lipid-specific or the glycoprotein-specific reagents used, but the mucuous (goblet) cells of all reference and exposed groups were PAS positive. Although PAS is highly reactive with glycoproteins, not all of these compounds give a positive reaction (Jones and Reid, 1978). In addition, different cell types synthesize various types of mucous glycoproteins which, in turn, respond differently to a variety of stimuli, from both hormones and xenobiotics (Parke, 1978). If rapid synthesis of abnormal mucus occurs, the typical staining reactions could be absent, because it has been shown that only when all the constituent residues are finally assembled in the glycoprotein moiety do they produce the colored PAS-reaction product (Reid and Clamp, 1978).

Changes in the chemical and physical properties of mucins in pathological states have been reported by Schrager and Oates (1978). They found that human gastrointestinal mucus from ulcer and cancer patients had altered rheological properties that may reduce the effectiveness of the mucous barrier against bacterial and enzymatic attack.

The persistence of the inclusions beyond the time of ingestion of the contaminants is consistent with the "limited return toward preexposure morphology" in the liver of rats exposed to polychlorinated biphenyls (Kasza et al., 1978) and may indicate degenerative changes beyond any possibility of recovery. Retention of chlorinated biphenyls beyond the time of exposure has also been reported in rainbow trout (Lieb et al., 1974) and in coho salmon (Gruger et al., 1975); information on the structure of the intestine from these fish is not available for comparison with the chinook salmon.

Based on the observation of similar weight gains of all groups during this study (Gruger et al., 1977b), functional impairment was not detected in the chinook salmon exposed to petroleum or chlorinated biphenyls. In other work, Gruger et al. (1976) showed that juvenile coho salmon gained weight when fed 1-12 ppm of a mixture of three chlorinated biphenyls in OMP. Mayer et al.

(1977) also found continued growth in coho salmon fed Aroclor 1254, a far more complex mixture of chlorinated biphenyl isomers than we used; however, all the fish fed a dose of 14,500 μg/kg body wt per day died after 265 days' exposure. The cause of death was not ascertained. Therefore, the observation in our studies of weight gain by the group of exposed fish showing obvious intestinal damage does not rule out the possibility that a degree of functional impairment existed which may have reduced long-term survival.

Summary

Sections of skin, gill, intestine, liver, and kidney were examined from juvenile chinook salmon exposed in parallel experiments to a model mixture of petroleum hydrocarbons, chlorinated biphenyls, and the combined contaminants. Morphological changes were observed only in liver and intestine, with changes in liver limited to salmon treated with chlorinated biphenyls. The most consistent and notable changes were in the mucosal cells of the intestine. The mucosa was intact in the control and petroleum hydrocarbon-exposed fish; some exfoliation was observed in the group exposed to chlorinated biphenyls. The group fed the combined contaminants (hydrocarbons and chlorinated biphenyls) had considerably increased sloughing indicating an interactive effect. The "goblet" or mucous cells appeared normal in all groups, but in the contaminant-exposed groups the columnar cells of the mucosa had distinct subcellular inclusions. The inclusions were not stained by carbohydrate-specific (PAS) or lipid-specific (Sudan black) reagents. Ultrastructurally, the inclusions that appeared after exposure to the hydrocarbons were variable in size and contained a flocculent, finely granular material. In the hydrocarbon-exposed group the inclusions differed from both the chlorinated biphenyl and the combined-contaminant groups. The inclusions in the latter groups included large, irregularly shaped vesicles with relatively electron-transparent material and other vesicles with a range of electron densities. The cellular alterations were consistent within the exposed group and presented a distinct change from normal morphology. Since the average gain in weight for all groups was similar, we could not conclude, however, that functional impairment from intestinal damage had occurred.

INTERACTIONS OF POLYCYCLIC AROMATIC HYDROCARBONS AND HEAVY METALS ON ACTIVITIES OF ARYL HYDROCARBON HYDROXYLASE

Methods

Coho salmon and starry flounder were initially held for 10-14 days in 10°C seawater to which was added 200 ppb of cadmium or lead, in order to allow time for heavy metals to interact with the hepatic AHH systems in the exposed fish. During the subsequent final two weeks, while the metal exposures continued, the fish were fed OMP which contained a model mixture of PAH's. The PAH mixture in the OMP consisted of 107 ppm (w/w) phenanthrene, 128 ppm 2-methylnaphthalene, and 99 ppm 2,6-dimethylnaphthalene. After the final two weeks, the fish were sacrificed and livers taken for analyses of AHH activities in microsomal preparations. The controls were fish that received an OMP diet without the addition of the PAH mixture. The experiment was carried out twice with the salmon and once with the flounder. The AHH activities were determined on six groups of seven to ten fish per group for each species. Activities of the AHH, as naphthalene hydroxylase, were determined by the method of Nilsson et al. (1976).

An assessment of the effects of the heavy metals on the microsomal AHH activities in an in vitro system was made. Cadmium and lead concentrations were separately varied from 0.05 to 50.0 ppm in enzymic reactions. The sources of microsomes were pooled samples of the liver fractions from the coho salmon which were fed chlorobiphenyls and hydrocarbons in the chronic exposure studies (discussed above) and from hatchery-reared rainbow trout.

Results and Discussion

The study of possible effects of the three dietary PAH's in coho salmon and starry flounder exposed to cadmium and lead in seawater showed little or no significant alterations in hepatic aryl hydrocarbon (naphthalene) hydroxylase activity for either species. The results given in Table 19 indicate that metabolic hydroxylations of aromatic hydrocarbons may not be

TABLE 19. NAPHTHALENE HYDROXYLASE ACTIVITY IN VITRO OF LIVER MICROSOMES OF PAH-FED FISH EXPOSED TO CADMIUM AND LEAD IN SEAWATER [a]

	Naphthalene hydroxylase activity [b]		
Exposure group	Coho salmon		Starry flounder
	Expt. 1	Expt. 2	Expt. 3
Control	0.50±0.22 (7)	0.20±0.13 (8)	0.30±0.25 (10)
Cadmium	0.82±0.24 (4)	0.26±0.13 (8)	0.45±0.41 (6)
Lead	0.52±0.14 (10)	0.28±0.13 (9)	0.22±0.14 (9)

[a] Fish fed Oregon moist pellets (OMP) containing 128 ppm (w/w) of 2-methylnaphthalene, 99 ppm 2,6-dimethylnaphthalene, and 107 ppm phenanthrene, while 200 ppb of the metals were added to the seawater. The fish were exposed to the metals, as cadmium chloride and lead nitrate, for 14 days following a period of 10-14 days of preconditioning in the test aquaria. Control group received OMP without the three aromatic hydrocarbons.

[b] Napthalene hydroxylase activity, as nmoles of products/mg microsomal protein, in 20 min incubations at 25°C, with other conditions according to Nilsson et al. (1976). Number of samples in parentheses.

affected by cadmium or lead exposures under the test conditions. For instance, duplicate experiments with coho salmon, i.e., Expt. 1 and 2 in Table 19, indicated a slight ($0.05<P<0.10$) inductive effect of cadmium on the activity of naphthalene hydroxylase in the first experiment and no effect in the second.

Previous studies showed that dietary PAH's affected microsomal AHH activity in coho salmon (Gruger et al., 1977b). The addition of the heavy metals, without effects on the AHH in the present study, did not take into account controls without PAH exposures. Thus, it may be assumed that any effect of the three PAH's to increase the activity of AHH may have been negated by the heavy metals; however, additional experiments are necessary to determine such an effect.

Other experiments were carried out with cadmium and tritiated naphthalene in incubation mixtures with hepatic microsomal preparations from coho salmon and rainbow trout. The results, which are given in Table 20, showed that 4-5 ppm ionic cadmium caused 82-98% inhibition of naphthalene hydroxylation. Lead was likewise found to cause about 84% inhibition on the same AHH system *in vitro*, but at a concentration of 10 ppm, which was about twice the concentration of cadmium for similar inhibition. However, when the effect of metals are considered on an equal molar basis, there was about the same inhibitory effect on the AHH activity by cadmium and lead.

The present results suggest that 50-200 ppb of heavy metals in microsomal preparations will not affect the activity of the hepatic naphthalene hydroxylase system in fish, and thereby not alter the toxicity of PAH's in fish exposed to petroleum. Furthermore, very high concentrations of metals were required to cause substantial inhibition of AHH. Hence, concentrations of heavy metals at unrealistically high levels in environmental waters may be necessary in order to detect significant changes associated with hepatic AHH activities.

DETERMINATION OF ACTIVITIES OF ARYL HYDROCARBON MONOOXYGENASE USING THREE DIFFERENT SUBSTRATES

Materials

Biochemicals and benzo[a]pyrene were obtained from Sigma Chemical Company (St. Louis, Mo.). Naphthalene was purchased from Baker and Adamson (Morristown, N.J.), and 2,6-dimethylnaphthalene was obtained from Chemical Samples Company (Columbus, Ohio). Organic solvents and inorganic chemicals were of reagent grade. Samples for liquid scintillation spectrometry were dissolved in PCS (Amersham Corporation, Arlington Heights, Ill.). Low-density polyethylene vials of 16 ml capacity were purchased from Olympic Plastics (Los Angeles, Calif.).

Benzo[a]pyrene (5 Ci/mmol), 2,6-dimethylnaphthalene (2 Ci/mmol), and naphthalene (85 Ci/mol), labeled with tritium, were obtained from the Amersham Corporation. The substrates were protected from light during handling and were stored at -20°C under nitrogen. The labeled hydrocarbons were purified

TABLE 20. INHIBITION OF HEPATIC MICROSOMAL ARYL HYDROCARBON HYDROXYLASE (AHH) ACTIVITY BY CADMIUM CHLORIDE AND LEAD NITRATE *IN VITRO* [a]

Concentration of metal	Inhibition of AHH activity [b]		
	Cadmium exposure		Lead exposure
	Coho salmon	Rainbow trout	Rainbow trout
ppm (w/w)	Percent		
0.05	0	0	--
0.10	--	13	0
0.50	7	23	0
1.00	39	39	22
2.00	--	60	--
3.00	--	75	--
4.00	--	84	--
5.00	98	82	42
10.0	97	--	84
50.0	100	72	57

[a] AHH as naphthalene hydroxylase. Microsomal preparations used were from coho salmon which were fed 10 ppm of 1:1 mixture of chlorobiphenyls and petroleum hydrocarbons (Gruger et al. 1977b), and rainbow trout.

[b] Inhibition determined as percent of AHH activity without added metal.

by elution from a column of silica gel (Yang et al., 1976). After this purification step, all three substrates were found to be greater than 99% pure. The purity of the $[^{3}\text{H}]$benzo[a]pyrene and the $[^{3}\text{H}]$naphthalene was verified by HPLC; the purity of the 2,6-dimethyl$[^{3}\text{H}]$naphthalene was checked by TLC. A Packard model 3255 liquid scintillation spectrometer was used to measure the radioactivity of the labeled substrates and metabolic products. Stock solutions of $[^{3}\text{H}]$naphthalene were prepared in ethanol; stock solutions of $[^{3}\text{H}]$benzo[a]pyrene and 2,6-dimethyl$[^{3}\text{H}]$naphthalene were prepared in acetone.

Preparation of Microsomes--

Coho salmon, weighing 200-500 g, were used for these studies. The fish were killed by a blow to the head. The livers were removed immediately and transported to the laboratory on crushed ice. All subsequent steps were conducted in the cold. The combined livers were weighed, minced with scissors, and then homogenized with a Potter-Elvehjem homogenizer, using 4 ml of 0.25 M sucrose solution per gram of liver. The homogenate was centrifuged at 2600 x g for 10 min. The resulting supernatant was centrifuged at 15,000 x g for 20 min. Microsomes were sedimented from the second supernatant at 105,000 x g for 1 hr. The surface of each microsomal pellet was rinsed gently with 1 ml of 0.25 M sucrose, and the rinse was discarded. The microsomes were suspended with a Dounce homogenizer in a volume of 0.25 M sucrose equal to the original wet weight of the livers. The microsomal suspension was stored at -60°C. When maintained at this temperature, there was no loss in monooxygenase activity over 8 months. Microsomes were thawed only once, immediately before use. Protein concentration was measured by the method of Lowry et al. (1951).

Procedures

Enzyme Assays--

A single set of incubation conditions was employed for the assay of the three substrates. The conditions used were those found to be optimum for determination of benzo[a]pyrene monooxygenase activity of coho salmon microsomes (Gruger et al., 1977b). Our incubation conditions were similar to those used by other investigators for fish liver monooxygenases (Pedersen et al., 1976; Chambers and Yarbrough, 1976; Pohl et al., 1974). Our assays were conducted at 25°C for 20 min; in other respects the conditions were similar to those developed for mammalian enzymes (Hansen and Fouts, 1972; Nilsson et al., 1976; DePierre et al., 1975).

The total reaction volume was 1.12 ml, and the final concentrations were: tris buffer, pH 7.5, 55 mM; $MgCl_2$, 2.6 mM; and NADPH, 1.2 mM. Microsomal protein concentration was 0.7 mg/ml. Substrate concentrations used are given with the results. The labeled substrate solutions had the following specific activities: benzo[a]pyrene, 14.6 Ci/mol; 2,6-dimethylnaphthalene, 3.9 Ci/mol; and naphthalene, 2.0 Ci/mol. The volume of substrate solution added was 20 μl for all assays.

Blanks, which contained all reagents except NADPH, were included for each substrate level. For benzo[a]pyrene assays, the tests and blanks were run in triplicate. For assays of naphthalene and 2,6-dimethylnaphthalene monooxygenases, duplicate tests and a single blank gave reliable results. Enzyme activity represents the difference between product formation in the tests and that observed in the blank(s). All components, except the labeled substrate, were added to the reaction tubes and incubated for 10 min at 25°C. Then the substrate was added and the reaction was run for 20 min at 25°C with shaking at a rate of 130 oscillations per min. After the reaction was stopped, unreacted substrate was extracted from the reaction mixture. All three assays are based on determination of radioactivity remaining in the aqueous phase after the unreacted substrate had been extracted. Unmetabolized naphthalene or 2,6-dimethylnaphthalene was adsorbed onto polyethylene as described by Nilsson et al. (1976). Our results indicated that using a series of three, rather than two, polyethylene vials gave better removal of unreacted substrate. The effectiveness of the polyethylene adsorption procedure for removal of [^{3}H]naphthalene has been established (Nilsson et al., 1976). We have verified that the procedure is equally effective for removal of 2,6-dimethyl[^{3}H]naphthalene. We found that 95% of unreacted 2,6-dimethyl[^{3}H]naphthalene was removed by the first adsorption step; 99.8% was removed after the second step; and 99.98% was removed after the third step. Recovery of total labeled metabolites of 2,6-dimethylnaphthalene was better than 85%. For the benzo[a]pyrene assays, the unreacted substrate was extracted into hexanes after stopping the reaction with ethanolic base. The validity of this extraction procedure has been established (DePierre et al., 1975). We found that two extractions with hexanes gave lower and more reproducible blank values. In all of the assays 0.3 ml of the aqueous phase was used for liquid scintillation counting.

Calculation of V_{max} and Apparent K_m Values--

The results of the kinetics experiments were plotted and constants were calculated using a Wang 720C calculator with an automatic data plotter. All of the kinetic data reported here were plotted by both the double-reciprocal method attributed to Lineweaver and Burk (1934) and by the method of Hofstee (1952) as recommended by Dowd and Riggs (1965). For most assays, the Lineweaver-Burk graphs yielded excellent straight lines but considerable errors in estimations of maximum velocity (V_{max}) and Michaelis constant (K_m). The alternate plots (v against v/s) demonstrated greater departure from linearity, but proved to be more useful for judging the nature of variations from Michaelis kinetics. The Hofstee plots were used to calculate values for V_{max} and K_m that are presented below. The square of the correlation coefficient, r^2, is cited in the text to indicate the degree of reliability of linear correlation for the graphs presented.

Results and Discussion

Benzo[a]pyrene has been used to assess aromatic hydrocarbon metabolism in fish (Gruger et al., 1977a, b; Payne and Penrose, 1975; Pedersen et al., 1976), but there is evidence suggesting that it is only a minor constituent of crude oils (Brown and Weiss, 1978) and, therefore, is not the most appropriate choice for research on the effects of petroleum pollution on marine animals. Metabolism of 2-methylnaphthalene *in vivo* by rainbow trout has been reported (Melancon and Lech, 1978). Dimethylnaphthalenes are abundant in crude oils and refined petroleum products (Clark and Brown, 1977). Moreover, alkylated naphthalenes can be accumulated and metabolized by marine animals (Anderson et al., 1974; Roubal et al., 1978).

For these experiments, reaction rates observed for the coho salmon liver monooxygenases with the three substrates are presented in Table 21. The table includes data at the lowest and highest substrate concentrations employed, and at the substrate level which is closest to the apparent K_m. In the case of naphthalene, the highest substrate concentration used and the apparent K_m coincided at 300 μM. Reaction rates which we obtained when benzo[a]pyrene was used as substrate were closely comparable to those previously obtained in this laboratory (Gruger et al., 1977a, b) and to results reported for a species of lake trout (Ahokas et al., 1975). However, the maximum reaction rate we obtained for benzo[a]pyrene monooxygenase is one third of that reported by DePierre et al. (1975) and only one tenth of the level observed in rainbow trout (Pedersen et al., 1976). Similarly, our data for monooxygenase towards naphthalene indicate that our enzyme preparation was one third as active as the rat liver microsomes assayed by Nilsson et al. (1976).

Figure 15 is the plot of v against v/s for benzo[a]pyrene concentration ranging from 0.16 to 80 μM. Calculations from the data of figure 15 (r^2=0.974) yielded a value of 122 pmol/mg per min for V_{max} and 2.1 μM for the apparent K_m. This value is comparable to data obtained by others: 1.2 μM (Robie et al., 1976), 2.48 μM (Cumps et al., 1977), and 2.95 μM (Gurtoo and Campbell, 1970) for rat liver benzo[a]pyrene monooxygenase and 2.8 μM for the Chambers Creek strain of rainbow trout (Pedersen et al., 1976). In other instances our value is lower than some reported values (Schenkman et al., 1977).

Figure 16 (r^2=0.953) is the plot of v against v/s for 2,6-dimethylnaphthalene over a concentration range of 0.7 to 144 μM. Calculations from Fig. 16 gave V_{max}=244 pmol/mg per min and an apparent K_m=15.3 μM. The assay obeyed Michaelis kinetics over the broad concentration range, and the K_m indicated that the enzyme preparation has a lower affinity for 2,6-dimethylnaphthalene than it has for benzo[a]pyrene. We are unaware of comparable data for dimethylnaphthalenes used as substrates for monooxygenases.

Naphthalene was tested as substrate for the fish liver microsomes over a concentration range of 1.5 to 300 μM. The data indicated that naphthalene concentrations below 15 μM produced low reaction velocities: i.e., the graph of 1/v against 1/s gave a negative intercept at the ordinate. This kinetic behavior has been noted by other researchers (Cumps et al., 1977; Hansen and Fouts, 1972; Robie et al., 1976) for benzo[a]pyrene monooxygenase activity of microsomes prepared from livers of induced rats. When the data for naphthalene over the concentration range of 15 to 300 μM were plotted, values for V_{max} and K_m could be calculated. Figure 17 (r^2=0.956) is the graph of v against v/s over this 20-fold concentration range. V_{max} from figure 3 is 314 pmol/mg per min, and the apparent K_m is 300 μM. The apparent K_m is appreciably different from the figure of 70 μM reported for rat liver microsomes (Nilsson et al., 1976). It is possible that the discrepancy between the two values is a reflection of limitations in the assay system, but a species difference cannot be ruled out.

The apparent K_m values for the three substrates indicate that our preparation of coho salmon liver microsomes had a high affinity for benzo[a]pyrene, intermediate affinity for 2,6-dimethylnaphthalene, and low affinity for naphthalene. The affinity of the enzyme for naphthalene was sufficiently low that the K_m of 300 μM approaches the maximum solubility of this substrate in the reaction medium. This characteristic limits the usefulness of naphthalene as an alternative to benzo[a]pyrene for monooxygenase assays.

The use of 2,6-dimethylnaphthalene as a substrate for fish microsomal monooxygenases appears very attractive. The apparent K_m of 15.3 μM indicates good affinity of the enzyme for this substrate, and Michaelis kinetics applied over a 200-fold concentration range. Methylated naphthalenes are interesting substrates for research on monooxygenases since oxidative metabolism can occur at the methyl substituent(s) or on the aromatic rings (Kaubisch et al., 1972).

TABLE 21. ACTIVITIES OF COHO SALMON LIVER MONOOXYGENASES WITH THREE POLYCYCLIC AROMATIC HYDROCARBON SUBSTRATES

Substrate	Concentration	Activity
	μMol	pmol products/mg per min
Benzo[a]pyrene	0.16	8.8
	1.6	46.8
	80	127
2,6-Dimethylnaphthalene	0.7	10.4
	14.4	84.2
	144	212
Naphthalene	1.5	1.2
	300	156

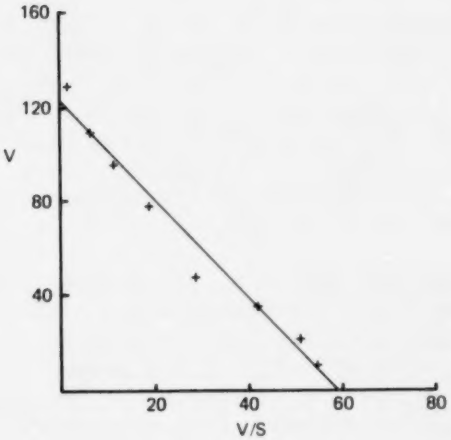


FIGURE 15. Hofstee plot of benzo[a]pyrene metabolism by liver microsomes of coho salmon. Abscissa represents the reaction velocities expressed as pmol products per mg protein per min divided by the benzo[a]pyrene concentration (0.16 to 80 μM) and the ordinate represents reaction velocities.

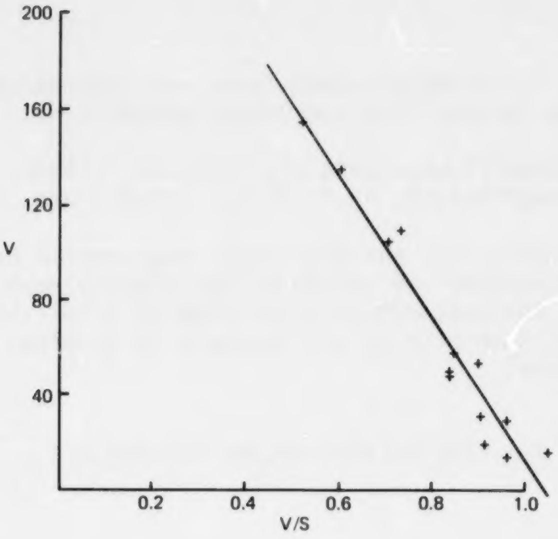


FIGURE 16. Hofstee plot of 2,6-dimethylnaphthalene metabolism by liver microsomes of coho salmon. Abscissa represents the reaction velocities expressed as pmol products per mg protein per min divided by 2,6-dimethylnaphthalene concentrations (0.7 to 144 μM) and the ordinate represents reaction velocities.

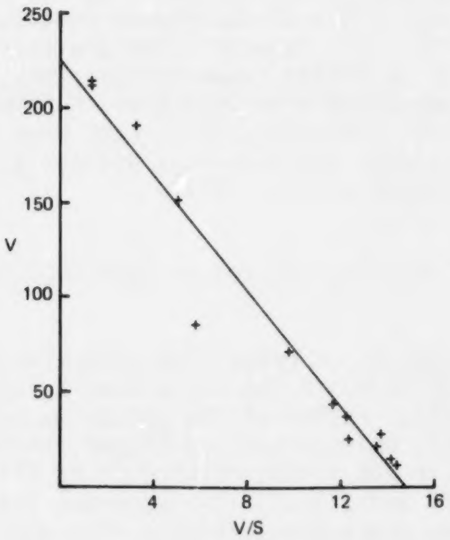


FIGURE 17. Hofstee plot of naphthalene metabolism by liver microsomes of coho salmon. Abscissa represents reaction velocities expressed as pmol products per mg protein per min divided by naphthalene concentrations (15 to 300 μM) and the ordinate represents reaction velocities.

Summary

Benzo[a]pyrene, 2,6-dimethylnaphthalene, and naphthalene were used as substrates for a coho salmon liver microsomal preparation.

The apparent Michaelis constants (K_m) were as follows: benzo[a]pyrene, 2.1 μM; 2,6-dimethylnaphthalene, 15.3 μM; and naphthalene, 300 μM.

The results indicate that the microsomal preparation had a high affinity for 2,6-dimethylnaphthalene. We conclude that dimethylnaphthalenes are important substrates for research on metabolism of alkyl-substituted PAH's in marine fish, and that such studies are relevant to problems of petroleum pollution of the oceans.

ORGANIC SYNTHESES OF 2,6-DIMETHYLNAPHTHALENE DERIVATIVES

Methods

Instrumental Analyses--

Proton NMR spectra were recorded on a Varian model CFT-20 spectrometer using tetramethysilane as an internal standard. Gas chromatography/mass spectrometry (GC/MS) analyses were performed on a Finnigan model 3200 automated gas chromatography/mass spectrometer operated at 70 eV. Other GC analyses were done on a Nuclear-Chicago model series 5000 gas chromatograph using a packed column with OV-101, 10% on 80-100 mesh Chromosorb G. High-pressure liquid chromatography (HPLC) was performed on a Spectra-Physics Model 3500 chromatograph equipped with a UV absorbance detector. Melting points were determined on a Fisher-Jones apparatus and are corrected. Elementary analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. 2,6-Dimethylnaphthalene was purchased from both Aldrich Chemical Co. (Milwaukee, WI) and Chemical Samples Co. (Columbus, OH). Fast Blue Salt B (azoicdiazo component 48) (Matheson Coleman and Bell) was used as a spray reagent for the detection of naphthols (Roubal et al., 1977).

Organic Syntheses--

The preparation and characterization of compounds (1-7, Fig. 18) are described below.

2,3-Dimethyl-3-naphthol (1)--Crystalline potassium 2,6-dimethylnaphthalene-3-sulfonate was prepared in 39% yield from 2,6-dimethylnaphthalene (Fieser and Seligman, 1934). Fusion of the potassium sulfonate with KOH (Fieser and Seligman, 1934; Weissgerber and Kruger, 1919) gave the white crystalling naphthol (1) in 50% yield, melting point 170-174° [literature mp 169-170° (Cassebaum, 1957) and 173-174° (Weissgerber and Kruber, 1919)]. TLC showed the presence of two trace contaminants. The purity of the naphthol (1) was estimated by gas chromatography (GC) to be 98%. The mass spectrum of the 98% component was in accord with the assigned structure.

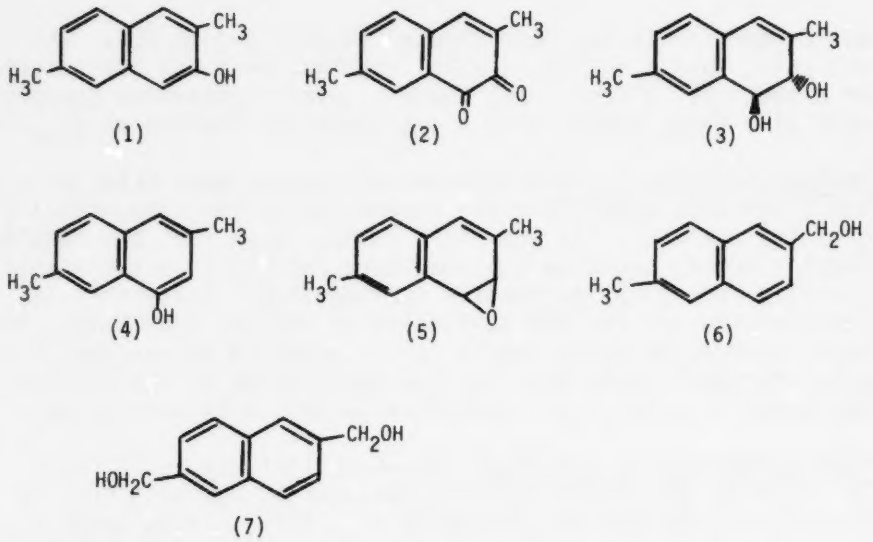


FIGURE 18. Oxygenated derivatives of 2,6-dimethylnaphthalene. (1), 2,6-dimethyl-3-naphthol; (2), 2,6-dimethyl-3,4-naphthoquinone; (3), trans-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene; (4), 2,6-dimethyl-4-naphthol; (5), 2,6-dimethylnaphthalene 3,4-oxide; (6), 6-methyl-2-naphthalenemethanol; (7), 2,6-naphthalenedimethanol.

2,6-Dimethyl-3,4-naphthoquinone (2)--The naphthoquinone (2) was prepared batchwise from (1) through the use of potassium nitrosodisulfonate (Fremy salt) (Cassebaum, 1957). Average yield of (2) was 79%. All batches had sufficient purity (98.1 to 99.6% by GC on OV-101) for use in the next synthetic step.

A batch was prepared, for use in Ames tests for mutagenicity (see next Section), by recrystallization from ethanol. Highly crystalline orange needles were obtained, mp 151.0-151.4^{o} (mp 151^{o}, Cassebaum, 1957). This sample was chromatographically homogeneous (GC on OV-101), and had a mass spectrum in accord with the assigned structure. Subsequently, a sizeable impurity (ca. 7%) was observed to be present by HPLC analysis. The accompanying product could not be removed by repeated recrystallization of the sample from ethanol. It is probably very closely related structurally to the quinone, since even on HPLC its retention time is nearly like that of the quinone. The accompanying product is a potential metabolite, and further investigation of its structure is important for the interpretation of the HPLC metabolic profiles and future Ames test results.

trans-3,4-Dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene (3)--The naphthoquinone (2) was reduced by an established synthetic route (Booth et al., 1950) with lithium aluminum hydride and gave, after workup and recrystallization, a 34% yield of (3) as white needles, mp 107.2-108.2^{o}.

Chromatographic homogeneity was demonstrated on HPLC and on TLC. The elemental analysis, mass spectrum, and NMR spectrum were all consistent with the assigned structure. NMR spectral features were furthermore supportive of the assignment of a trans rather than a cis structure (Jerina et al., 1971).

2,6-Dimethyl-4-naphthol (4)--Different procedures were tried on a small scale to find a workable method for the conversion of the dihydrodiol (3) to the mixture of the naphthols (1) and (4). It was found that the naphthol (4), the predominant product, could be sublimed more readily than the naphthol (1) and thus separated from it by successive sublimations. Analytical TLC procedures were worked out for the separation of the two naphthols. Both naphthols were found to be quite labile in the presence of air and light. All of the preliminary small scale work for the preparation of the naphthol (4) has been completed; a larger scale synthesis is yet to be carried out.

2,6-Dimethylnaphthalene 3,4-Oxide (5)--A preliminary small scale synthesis of (5) from the dihydrodiol (3) was carried out with the use of dimethylformamide dimethylacetal (Harvey et al., 1975). Mass spectral confirmation of the formation of (5) was obtained. In the presence of an added acid catalyst, (5) very rapidly rearranged to a naphthol. With careful exclusion of traces of acid, spontaneous rearrangement and loss of (5) still took place (in solution at -20°), but the process was slowed.

6-Methyl-2-naphthalenemethanol (6)--White solid 2-bromomethyl-6-methylnaphthalene was prepared from 2,6-dimethylnaphthalene according to literature procedures (Buu-Hoi and Lecocq, 1946; Bullpitt et al., 1976). The bromide obtained was highly impure. Attempts to upgrade the purity of the somewhat unstable sample by recrystallization did not work well. Thus, the impure bromide was reacted with acetone, water, and silver nitrate to give (6) in addition to several by-products. Compound (6) was purified by preparative TLC and had mp 131.7-132.7° (mp 128-130; Julia et al., 1960). NMR and mass spectra were in accord with the assigned structure. With any exposure to air and light, a major decomposition product (faster migrating on TLC) was observed to arise directly from the alcohol (6). This conversion is of interest, since the decomposition product may be involved metabolically.

2,6-Naphthalenedimethanol (7)--White solid 2,6-bis(bromomethyl)naphthalene was prepared from 2,6-dimethylnaphthalene according to literature procedures (Ried and Bodem, 1958; Golden, 1961). The dibromide was to be converted to 2,6-bis(acetoxymethyl)naphthalene, which may then be converted to (7) (Storms and Taussig, 1966); however, low yields and impurities in the intermediates resulted in postponing further work on the dimethanol (7).

Preparation of Samples for Mutagenesis Tests--

Compounds (1)-(3) and (6) were prepared in sufficient quantity (125 mg required) and purity for use in the Ames test for mutagenesis (Ames et al., 1975; cf., next Section).

MUTAGENESIS ASSAYS OF 2,6-DIMETHYLNAPHTHALENE DERIVATIVES

Methods

The Ames assays for mutagenicity were carried out in duplicates on the following chemicals: 2,6-dimethylnaphthalene, 2,6-dimethyl-3-naphthol, 2,6-dimethyl-3,4-naphthoquinone, *trans*-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene, 6-methyl-2-naphthalenemethanol, and *trans*-1,2-dihydroxy-1,2-dihydronaphthalene. Tests on known carcinogens, 2-aminoanthracene and benzo[a]pyrene, were carried out for comparison with the naphthalenic compounds.

Agar plate assays were carried out essentially as decribed by Ames et al. (1975). Dimethylsulfoxide was used as solvent for all of the chemicals, which were tested at eight concentrations in the range of 2-1,000 μg/plate. To obtain maximum expression of mutagenicity, agar plates were preincubated to a temperature of 37°C before adding the components of the Ames assay to the semisolid agar. After the top-agar layer was poured onto the 37°C plates, they were incubated in the upright position for several hours to allow hardening of the semisolid top-agars. The assay plates were then inverted and incubated for 24-36 hr to allow expression of mutant colonies. Colonies originated from TA98 and TA100 strains of *Salmonella typhimurium*. Colonies were counted using a Biotran II automated colony counter (New Brunswick Scientific Co., Inc., Edison, NJ).

Liver homogenates used in the mutagenicity assays were prepared from male Wistar rats, which had been injected intraperitoneally with 250 mg/kg body weight of a polychlorinated biphenyl (Aroclor 1254, Monsanto Chemical Corp.) to provide the necessary induced mixed-function oxidase system. After sacrifice, the livers were perfused with ice-cold 0.154 M KCl and removed for preparation of an S9 homogenate (9,000 x g supernate), following standard procedures (Ames et al., 1975).

Another series of tests for mutagenesis were carried out with phage-induction procedures described by Moreau et al. (1976). The tests used *E. coli* K12 permeable (envA) tester bacteria, which is deficient in DNA repair (uvrB). These "induct tests" were carried out on the chemicals at 20, 40, and 100 μg/plate, in quadruplicates with and without the S9 liver homogenate.

Results and Discussion

The responses of TA98 and TA100 cells, in numbers of revertant colonies per plate, for the naphthalenic compounds were essentially all negative. The data for the three largest concentrations of each compound are presented in Tables 22 and 23. Only 2,6-dimethyl-3,4-naphthoquinone showed an effect by killing the cells at 100 μg/plate with 810 μg S9 homogenate; killings were observed also at 500 μg/plate with 135 μg of S9 homogenate. The reference compounds, 2-aminoanthracene and benzo[a]pyrene, which gave positive responses at 0.5 μg/plate and 5 μg/plate, respectively, were not tested at other concentrations to determine if similar killings were possible.

TABLE 22. TESTS FOR MUTAGENIC ACTIVITY IN *S*. *TYPHIMURIUM* TA-98 CELLS

Compound tested	Amount tested	Ratio of response-to-background (Response) [a]		
		0 µg S-9	135 µg S-9	810 µg S-9
	µg/plate			
2,6-Dimethylnaphthalene	100	0.97 (114)	1.29 (27)	0.53 (15)
	500	1.17 (132)	0.62 (13)	0.67 (19)
	1,000	0.98 (116)	1.33 (28)	0.46 (13)
2,6-Dimethyl-3-naphthol	100	1.03 (122)	0.67 (14)	0.98 (25)
	500	0.92 (109)	0.71 (14)	1.12 (32)
	1,000	0.98 (116)	1.24 (26)	---
2,6-Dimethyl-3,4-naphthoquinone	20	---	0.86 (18)	0.67 (19)
	100	0.87 (103)	(killing)	1.47 (42)
	500	0.99 (117)	(killing)	(killing)
trans-3,4-Dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene	100	0.86 (102)	0.62 (13)	0.74 (21)
	500	0.97 (114)	1.52 (32)	0.81 (23)
	1,000	1.10 (130)	1.19 (25)	0.84 (24)
6-Methyl-2-naphthalenemethanol	100	1.07 (126)	1.48 (31)	0.56 (16)
	500	0.87 (103)	0.90 (19)	0.95 (27)
	1,000	---	0.90 (19)	0.56 (16)
trans-1,2-Dihydroxy-1,2-dihydronaphthalene	100	0.86 (101)	0.76 (16)	0.91 (26)
	500	1.23 (145)	1.48 (31)	1.30 (37)
	1,000	---	1.50 (22)	0.49 (14)
2-aminoanthracene	0.5	---	49.7 (1,416)	---
	0.5	0.85 (100)	38.8 (814)	---
Benzo[a]pyrene	5	---	---	28.9 (675)
	5	1.07 (126)	---	25.7 (606)
None	---	1.00 (118)	1.00 (21)	1.00 (28)

[a] Response, in parentheses, is the average number of histidine revertant colonies per plate.

TABLE 23. TESTS FOR MUTAGENIC ACTIVITY IN S. TYPHIMURIUM TA-100 CELLS

Compound tested	Amount tested	Ratio of response-to-background (Response) [a]	
		135 µg S-9	810 µg S-9
	µg/plate		
2,6-Dimethylnaphthalene	100	0.83 (119)	1.42 (142)
	500	0.93 (133)	1.13 (113)
	1,000	0.56 (80)	1.06 (106)
2,6-Dimethyl-3-naphthol	100	0.76 (108)	0.85 (85)
	500	0.81 (116)	1.27 (127)
	1,000	0.92 (131)	0.90 (90)
2,6-Dimethyl-3,4-naphthoquinone	20	0.44 (63)	0.65 (65)
	100	0.49 (70)	(killing)
	500	(killing)	(killing)
trans-3,4-Dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene	100	0.92 (132)	1.05 (105)
	500	0.52 (75)	0.92 (92)
	1,000	0.76 (108)	0.65 (65)
6-Methyl-2-naphthalenemethanol	100	0.71 (102)	1.14 (114)
	500	0.63 (90)	1.28 (128)
	1,000	0.44 (63)	0.69 (69)
trans-1,2-Dihydroxy-1,2-dihydronaphthalene	100	0.83 (119)	0.86 (86)
	500	0.59 (81)	0.91 (91)
	1,000	0.52 (75)	0.84 (84)
2-aminoanthracene	0.5	6.85 (980 ± 136)	---
	0.5	10.81 (1081 ± 260)	---
Benzo[a]pyrene	5	---	4.99 (714 ± 213)
	5	---	4.70 (470 ± 100)
None	--	1.00 (143 ± 17)	1.00 (100 ± 21)

[a] Response, in parentheses, is the average number of histidine revertant colonies per plate.

The results of the induct tests with E. coli K12 cells and the naphthalenic compounds are illustrated by Figure 19, where test-response to background-response ratios for these compounds can be compared with results with reference carcinogens. Again, 2,6-dimethylnaphthalene and the naphthalenic derivatives gave negative results from the induct test.

Assuming that the microbiological test data can be extrapolated to fish, then none of the naphthalenic compounds which were tested would be likely to cause changes that are mutagenic in fish. Of those compounds, 2,6-dimethyl-3,4-naphthoquinone appears to be the most potentially lethal to isolated cells at the concentrations employed in the assays. Work on toxic effects of naphthalenic quinones in fish should be considered for the future. Quinones are conversion products of phenolic compounds, which can be formed by free-radical autoxidations of various substituted aromatic hydrocarbons in the presence of oxygen (Uri, 1961); consequently, quinones in fish may arise from exogenous as well as endogenous reactions of petroleum aromatic hydrocarbons.

Summary

Tests were conducted on several naphthalenic compounds for their potential mutagenic activities. An Ames test with Salmonella was performed on each of the following: 2,6-dimethylnaphthalene, 2,6-dimethyl-3-naphthol, 2,6-dimethyl-3,4-naphthoquinone, 6-methyl-2-naphthalenemethanol, trans-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene, and trans-1,2-dihydroxy-1,2-dihydronaphthalene. 2-Aminoanthracene and benzo[a]pyrene, as references, were the only compounds that gave positive results with the Ames test. A lethal effect upon the Salmonella caused by 2,6-dimethyl-3,4-naphthoquinone was the only effect found for the naphthalenics tested. The research results suggest that, as far as can be generalized from microbial bioassays, mutagenesis is an unlikely event in fish due to exposures to these naphthalenic substances.

IN VITRO METABOLISM OF 2,6-DIMETHYLNAPHTHALENE BY COHO SALMON LIVER MICROSOMES

Methods

Microsomal Reactions In Vitro and Analyses--

Microsomes from normal coho salmon livers were prepared by homogenization and differential centrifugation. The microsomes were incubated at 25°C with 2,6-dimethylnaphthalene (2,6-DMN) in a reaction mixture consisting of Tris buffer pH 7.5, $MgCl_2$, and reduced nicotinamide adenine dinucleotide phosphate (NADPH). Controls contained everything except the NADPH. The concentration of 2,6-DMN was usually 30.6 μM. This value is twice the Michaelis constant for this substrate (cf. Table 21, above) (Schnell, Gruger and Malins, 1980). Unreacted substrate and nonconjugated metabolites were extracted into ethyl acetate from the reaction mixture at pH 7.5. Conjugated metabolites were then extracted into ethyl acetate after the aqueous phase was adjusted to pH 1 with hydrochloric acid.

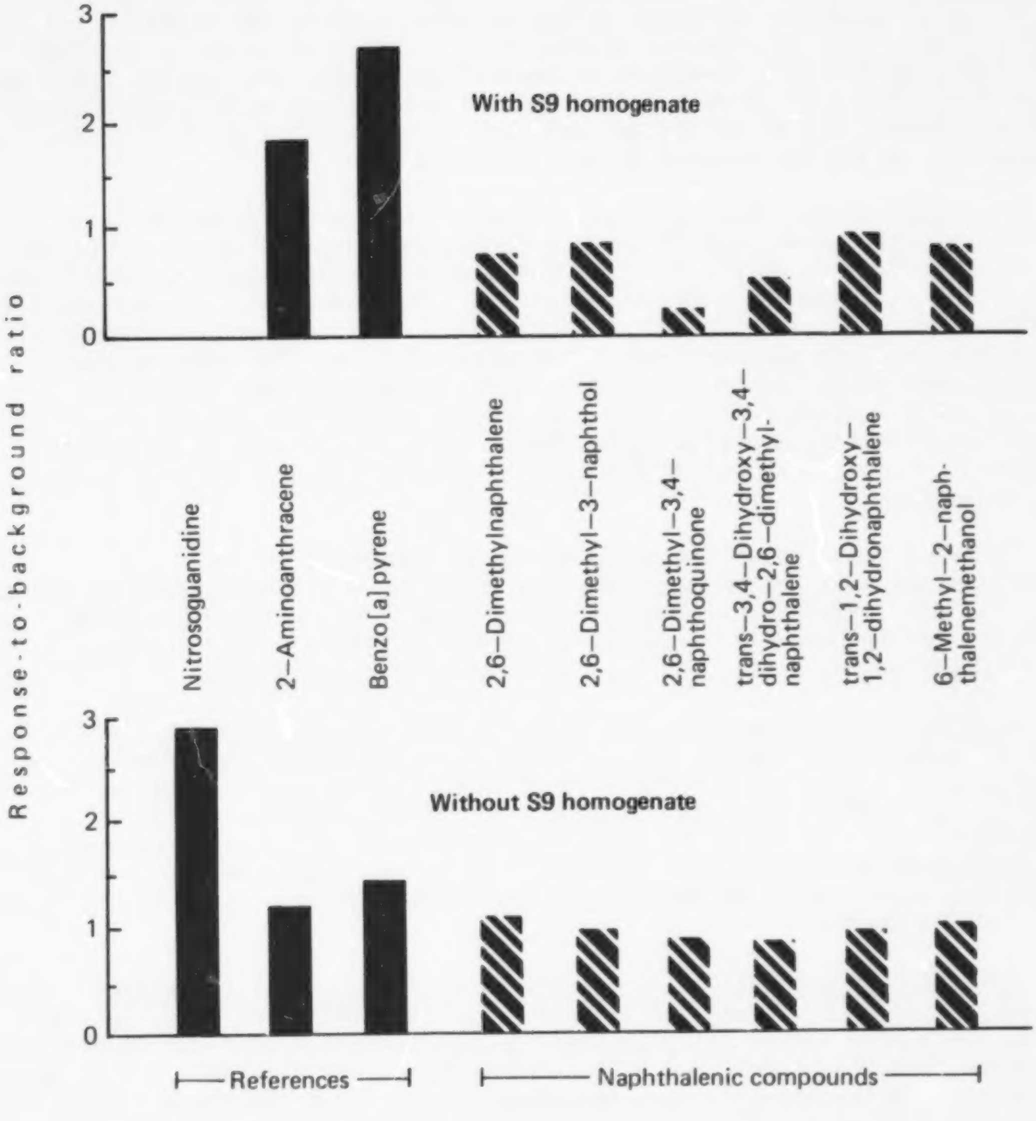


FIGURE 19. Induct test response-to-background ratios for naphthalenic compounds (100 μg/plate) and reference compounds (1 to 10 μg/plate) with *E. coli* K12 cells.

For preparation of the glucuronides of oxidized products of 2,6-dimethylnaphthalene, we employed conditions similar to those reported by Nilsson et al. (1976). After 30 min. incubation, Triton-X-100 and uridine diphosphoglucuronic acid were added. The incubation was then resumed for an additional 30 minutes. An equal volume of ethyl acetate was added to stop the reaction and to extract neutral metabolites. A total of three extractions with ethyl acetate were conducted at neutral pH. Then, the aqueous phase was adjusted to pH 1 with 1M HCl, and three more extractions with ethyl acetate were performed. TLC on silica gel was used to establish a profile of the metabolites in the two organic solvent extracts.

The metabolites, which were extracted at pH 1, were subjected to hydrolysis with limpet β-glucuronidase (containing aryl sulfatase activity) as described by Dodgson et al. (1953). Inhibition of sulfatase was accomplished by the method of Dodgson and Spencer (1953). The hydrolysis was conducted at pH 4.0 in acetate buffer at 37°C for one hour. The reaction was stopped and hydrolysis products were extracted with ethyl acetate. The ethyl acetate extracts were subjected to TLC on silica gel using a solvent system of toluene:ethyl acetate (50:10; v:v). Potential metabolites of 2,6-dimethylnaphthalene, prepared by Dr. Peter Fraser (cf., section on organic syntheses, above), were run in the same solvent system and their Rf values were determined.

Analyses for metabolites of 2,6-DMN were conducted by gas chromatography/mass spectrometry (GS/MS), thin-layer chromatography on silica gel, and high-performance liquid chromatography (HPLC) using ultraviolet fluorescence spectrometry (Krahn et al., 1980). Liquid scintillation spectrometry was used to assay for 2,6-dimethyl[^{3}H]naphthalene.

Analyses of 2,6-dimethylnaphthalene metabolites by glass-capillary GC/MS--Samples were injected into a Hewlett-Packard 5840A gas chromatograph interfaced to a Finnigan 3200 mass spectrometer. The GC column was a 30 m x 0.25 mm (i.d.) WCOT capillary column coated with SE-54 (Supelco, Inc.; Bellafonte, PA). The injector temperature was 320°C and column temperature was programmed at 4°C/min from 50-280°C. Mass spectrometer/electron impact conditions were: electron energy, 70eV; filament emission current, 500 A; and scan, 34-534 Amu/sec.

Detection of metabolites of 2,6-dimethylnaphthalene by thin-layer chromatography--TLC was used to monitor metabolite formation on a routine basis. We employ silica gel plates and a dual solvent system for development. The plates are first developed with ethyl acetate to a distance of 1 cm above the origin. This serves to move the dihydrodiol of 2,6-DMN cleanly away from the origin. After a brief drying, the plate is then developed with toluene:ethyl acetate (40:1, v:v). This system provides good resolution of the nonconjugated metabolites. Conjugated metabolites and highly-polar metabolites (e.g., more polar than dihydrodiol and monocarboxylic acids) will remain at the origin in this system.

Time Course for *In Vitro* Metabolism--

For a time course experiment, the incubation procedure was modified slightly. The usual volumes were increased to allow for analyses of sufficient numbers of reaction samples.

Test and control were run simultaneously; the control had no NADPH. At selected times (Table 24), an aliquot was removed from each reaction mixture and immediately extracted with ethyl acetate. The extracts and marker compounds were analyzed by TLC. Metabolites were located on TLC plates by scraping segments and counting them by liquid scintillation spectrometry. The radioactivity in the metabolite fractions from TLC was corrected for the slight activity found in the zero time sample.

Results

GC/MS analyses confirmed the presence of several metabolites prepared by *in vitro* incubation with microsomes. We have obtained confirmation of the substrate 2,6-DMN and the following metabolites: 6-methyl-2-naphthalenemethanol, two naphthols of 2,6-DMN, and a quinone of 2,6-DMN. Also, we have a tentative confirmation of the presence of 6-methyl-2-naphthaldehyde. None of the metabolites were detected in the control sample.

Thin-layer chromatography of neutral metabolites indicates that 6-methyl-2-naphthalenemethanol is a significant metabolite of 2,6-DMN by coho salmon hepatic microsomes. Conditions designed to prepare glucuronides of the polar metabolites indicate that we are able to form a product, which is extracted into ethyl acetate at pH 1 but not at pH 7. Hydrolysis of the acidic metabolites with β-glucuronidase (sulfatase inhibited with phosphate) liberates at least three significant metabolites of 2,6-DMN.

Data from the time-course experiment are given in Tables 24 and 25. The results provided considerable information on the nature of 2,6-DMN metabolism by fish liver microsomes. In Table 25, data are given that summarize the metabolite distribution at 15 minutes of incubation with the microsomes, and the Rf values for the marker compounds. These data indicate that the principal metabolite formed during the incubation was 6-methyl-2-naphthalenemethanol. Also, an aldehyde of 2,6-DMN was apparently a major metabolite. Lesser amounts of a quinone, the dihydrodiol, and a naphthol(s) were also detected. The Rf values indicate that the TLC system provides good separation of the compounds.

In A and B of Figure 20, plots are shown for the time course of the formation of total metabolites and individual metabolites. It can be seen that oxidation of the substrate occurs in a linear fashion for the first twenty minutes. After this time, the rate decreases, but metabolite formation still proceeds at a significant rate after 60 minutes. Also, there are important differences in the rates of formation of the identified metabolites. At all time points, the principal metabolites were the 6-methyl-2-naphthalenemethanol and the corresponding aldehyde. The results for the formation of the dihydrodiol were not plotted, because its formation followed an irregular time course. The formation of the naphthol(s) of 2,6-DMN was not very extensive in this experiment and seemed to cease after the first 15 minutes.

TABLE 24. TIME COURSE OF METABOLITE FORMATION FROM 2,6-DIMETHYL[^{3}H]NAPHTHALENE

Time	Total metabolites	Arylmethyl alcohol	Aldehyde	Dihydrodiol	Quinone	Naphthol
min	- - - - - - - - - - -	dpm / unit volume	of reaction mixture	- - - - -		
5	5,342	2,420	1,227	271	371	222
10	9,341	3,746	2,412	447	659	392
15	15,302	5,551	3,570	1,227	1,015	767
20	19,089	6,871	4,593	1,325	1,340	772
30	23,066	8,638	5,422	2,004	1,494	664
45	28,076	10,048	7,656	2,238	1,811	621
60	33,573	12,249	9,014	3,235	2,072	851

TABLE 25. METABOLITES OF 2,6-DIMETHYLNAPHTHALENE FROM IN VITRO INCUBATIONS OF 15-MINUTE REACTION WITH LIVER MICROSOMES

Compound	Percentage of total metabolites[a]	Percentage of identified metabolites	Rf[b]
2,6-Dimethylnaphthalene	---	---	0.94
Aldehyde	23.3	29.4	0.73
Naphthol	5.0	6.3	0.56
Quinone	6.6	8.4	0.47
Arylmethyl alcohol	36.3	45.8	0.32
Dihydrodiol	8.0	10.1	0.12

[a] 21% of dpm in areas of TLC bands were not positively identified.

[b] Rf values for marker compounds.

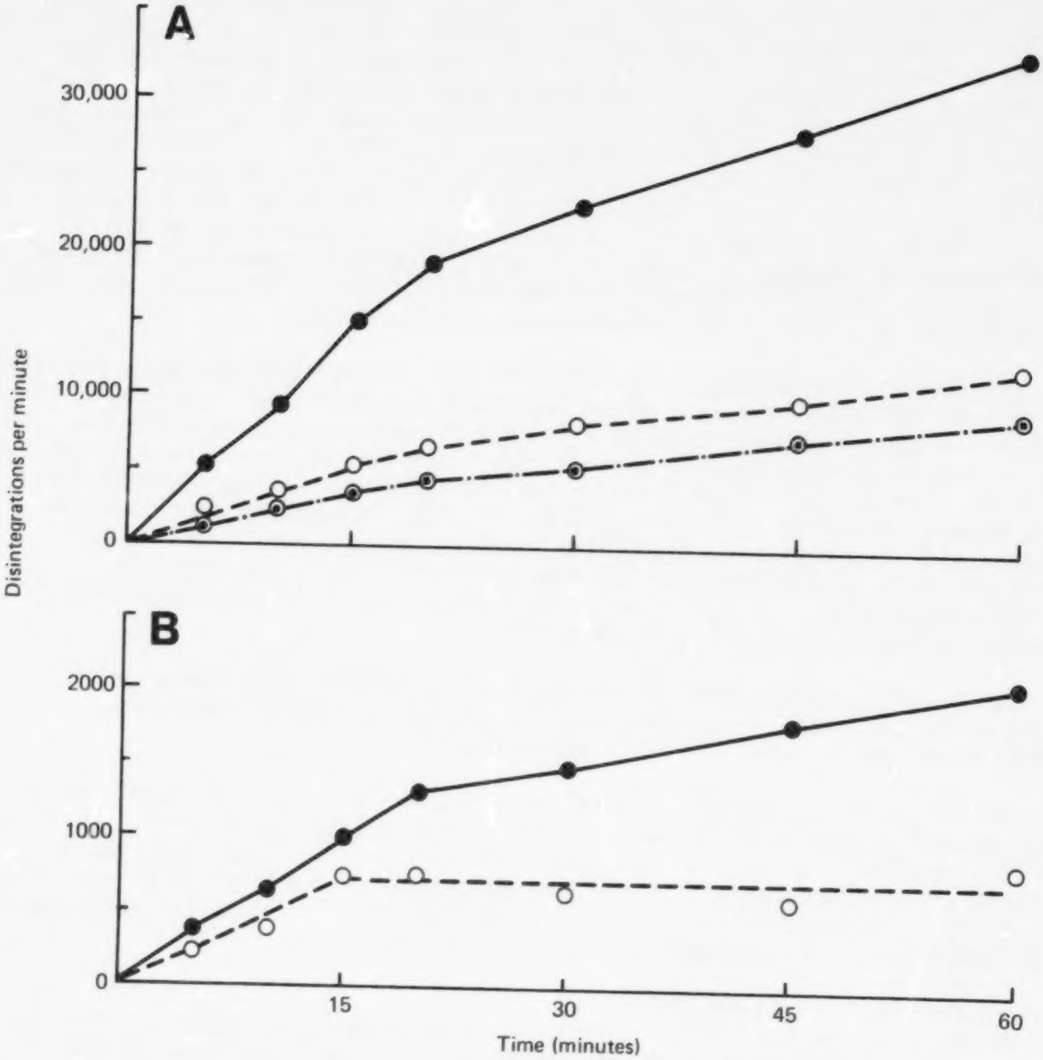


FIGURE 20. *In vitro* metabolism of 2,6-dimethylnaphthalene. A. Formation of total metabolites (———), 6-methyl-2-naphthalenemethanol (-----), and 6-methyl-2-naphthaldehyde (—·—·—). B. Formation of 2,6-dimethylnaphthoquinone (———) and 2,6-dimethylnaphthol (-----).

Discussion

The results of the GC/MS analyses verified some of our preliminary conclusions based on the results of TLC of ^3H-2,6-DMN metabolites formed in vitro. The results have provided proof that in vitro metabolism of 2,6-DMN results in the formation of 6-methyl-2-naphthalenemethanol. Ring oxidation of 2,6-DMN is indicated by confirmation of the presence of a quinone and two naphthols of the substrate. (The hydroxyl group could be on the 1, 3, or 4 position of the naphthalene moiety.) The quinone does not correspond to our standard of 2,6-dimethyl-3,4-naphthoquinone. In addition to ring-oxidation products, we believe an aldehyde is formed in the in vitro systems. Although GC/MS did not provide clear confirmation of the formation of the 6-methyl-2-naphthaldehyde, this compound is a logical product of oxidation of the 6-methyl-2-naphthalenemethanol, and it is of considerable interest. In other work, we found that ultraviolet irradiation of 2,6-DMN in air gave rise to a compound that was identified as 6-methyl-2-naphthaldehyde.

Thin-layer chromatography in two different solvent systems indicates that 6-methyl-2-naphthalenemethanol is an important metabolite of 2,6-DMN. Metabolites generated when uridine diphosphoglucuronic acid is added are readily separated from neutral metabolites by different extractions at pH 7 and pH 1. The acidic metabolites can be hydrolyzed by β-glucuronidase to yield several oxidized forms of 2,6-DMN. In the presence of saccharo-1,4-lactone, this hydrolysis is inhibited. One of the products released by hydrolysis with β-glucuronidase has the same Rf values as 6-methyl-2-naphthalenemethanol. At least two other metabolites of 2,6-DMN are also released by the β-glucuronidase hydrolysis.

Our results suggest that an aldehyde of 2,6-DMN is apparently an important metabolite. An aldehyde may be formed by further enzymatic oxidation of the 6-methyl-2-naphthalenemethanol.

Oxidation of the methyl group(s) of 2,6-DMN represents an important in vitro metabolic pathway involved in coho salmon hepatic microsomes. Oxidation of methylated naphthalenes at the alkyl substituents has been reported in bacteria (Starovoitov et al., 1976) and by guinea pig microsomes (Kaubisch et al., 1972). Thus, oxidation of alkyl substituents of methylated naphthalenes is a common metabolic pathway in a variety of organisms.

Interproject Collaborative Studies--

Metabolites of 2,6-DMN and benzo[a]pyrene, which were obtained from in vitro reactions with coho salmon liver microsomes, were employed by our National Analytical Facility, notably Dr. Margaret Krahn, in studies of HPLC. An aim of that work was to find out whether it was possible to detect the presence of the metabolites of 2,6-DMN among the metabolites of benzo[a]pyrene. The findings from this interproject collaboration have been submitted for publication (Krahn et al., 1981).

Summary

Our results indicate that coho salmon liver microsomes actively metabolize 2,6-DMN *in vitro*. The results indicated that methyl-group oxidation is the *principal* metabolic pathway for 2,6-DMN. An aldehyde and an alcohol arising from methyl-group oxidation are principal products of the metabolism *in vitro*; the former is believed to be 6-methyl-2-naphthaldehyde and the *latter is the* 6-methyl-2-naphthalenemethanol. Metabolic products of naphthyl-ring oxidations were isolated from the *in vitro* reactions, namely a quinone, two naphthols and a dihydrodiol of 2,6-DMN. A time-course study of the metabolites formed *in vitro* revealed limited formation of the naphthols.

EFFECTS OF NAPHTHALENE AND P-CRESOL, SEPARATELY AND TOGETHER IN FOODPATH EXPOSURES, IN STARRY FLOUNDER ON THE *IN VIVO* METABOLISM OF 2,6-DIMETHYLNAPHTHALENE

Materials

2,6-Dimethylnaphthalene-4-^{14}C (2.1 mCi/mmol) was synthesized by California Bionuclear Corp., Sun Valley, Calif., and found to be 98% radiochemically pure by gas-liquid chromatography. The following chemicals were obtained from commercial sources: 2,6-DMN (Aldrich Chemical Co., Milwaukee, Wisc., and Chemical Samples Co., Columbus, Ohio); potassium nitrosodisulfonate (K & K Laboratories, Plainview, N.Y.); Fast Blue Salt B (Matheson, Coleman and Bell, Norwood, Ohio); Amberlite XAD-2 and XAD-4 resins (Rohm & Haas Co., Philadelphia); limpet β-glucuronidase, α-glucosidase, β-glucosidase, α-naphthyl sulfate, β-naphthyl-α-D-glucoside and α-naphthyl-β-glucuronide (Sigma Chemical Co., St. Louis, Missouri); p-cresol (Aldrich Chemical Co.); and naphthalene (Baker and Adamson, Morristown, N.J.). All other chemicals were reagent grade, pesticide grade, or highest-available purity.

Four derivatives of 2,6-DMN were synthesized for use as reference standards. These compounds are described in a previous section; cf. derivatives 1, 2, 3, and 6 in Figure 18.

Starry flounder were netted from near the mouth of the Columbia River and acclimated to seawater holding facilities for six weeks. For an initial experiment, five fish were used. For a major experiment, forty-eight fish were selected for uniformity in size and randomly divided into four groups of twelve each. Of the latter fish, mean lengths and weights were as follows: group 1, 139±6 mm, 24±4 g; group 2, 150±10 mm, 27±6 g; group 3, 150±9 mm, 29±5 g; and group 4, 147±7 mm, 27±4 g. There were no significant differences ($P>0.05$) in length and weight among the four groups. The temperature of the water was 12.7±0.7°C and the salinity was 28.3±1.0°/oo. The flounder were given minced herring and hake *ad libitum* as a daily diet throughout the experiment.

Methods and Procedures

Instrumental Analyses--

Radioactivity was determined with a Packard Tri-Carb model 3255 liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.) using ScintiVerse scintillation fluid (Fisher Scientific Co., Fair Lawn, N.J.). HPLC of 2,6-DMN metabolites was performed on a Hewlett-Packard model 1084B chromatograph (Hewlett Packard Co., Palo Alto, Calif.) equipped with a 0.4 cm i.d. x 25 cm steel column packed with 10-μm diameter Lichrosorb RP-18 (E. Merck, Darmstadt, Germany). Gas chromatography/mass spectrometry was performed using a Hewlett-Packard 5840A gas chromatograph equipped with a glass capillary column (SE-54 wall coated), 30 meters in length, and interfaced with a Finnegan (Sunnyvale, Calif.) model 3200 automated mass spectrometer. Other GC analyses were performed on a Nuclear Chicago 5000 series gas chromatograph, using a column packed with 10% OV-101 on 80-100 mesh Chromosorb G (Applied Science Lab., State College, Penn.).

Thin-layer Chromatography--

Metabolites of ^{14}C-2,6-DMN were separated by TLC utilizing three different systems. For system A, silica gel 60 F-254 (E. Merck, Darmstadt, Germany) plates were developed with toluene:ethanol (92:8, v/v) in an unlined tank. For system B, silica gel Q6F (Kontes Glass Co., Vineland, N.J.) plates were first developed with toluene:ethyl acetate (85:15, v/v) to 15 cm in an unlined tank and then developed with ethanol:acetonitrile:acetic acid (90:10:1, v/v/v) to 8 cm in a filter paper-lined tank. For system C, Q6F plates were developed with toluene:acetone:acetonitrile (80:10:10, v/v/v) to 15 cm in a lined tank and then developed with n-butyl alcohol:water:ammonium hydroxide (85:13:2, v/v/v) to 8 cm in a lined tank.

Reference compounds consisted of 2,6-DMN, the four derivatives of 2,6-DMN (described above), α-naphthyl sulfate, α-naphthyl-β-glucuronide, and β-naphthyl-α-D-glucoside. Following TLC separations, the reference compounds were located on TLC plates using short wavelength ultraviolet light. The Rf values for the reference compounds in the three TLC systems are given in Table 26. Solvent system A was used for gross separations of conjugated metabolites from unconjugated metabolites; conjugated metabolites of 2,6-DMN remain at the origin. TLC system B resolved all of the reference compounds except for α-naphthyl sulfate and trans-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene. These two compounds could be effectively separated by the use of system C. All reference compounds were stable during the course of TLC analyses, although certain chemical changes were observed on TLC plates after 12-18 hrs. Recovery of radiolabeled metabolites from TLC plates was greater than 90%.

Exposure of Starry Flounder--

Two exposure experiments were conducted: the first was a small-scale experiment to determine the time intervals to be employed for the major, large-scale experiment. The initial experiment involved the administration of ^{14}C-2,6-DMN to five starry flounder. Each of these fish was fed 9.6 μCi (0.71 mg) of the labeled 2,6-DMN in a gelatin capsule containing Oregon moist pellet food. At 6, 12, 24, 48, and 120 hours after administering the capsules, a fish was killed, and the liver and gall bladder were removed for analysis. Analysis of radioactivities in the latter tissue indicated that

TABLE 26. Rf VALUES FOR REFERENCE COMPOUNDS ANALYZED BY THIN-LAYER CHROMATOGRAPHY USING THREE SOLVENT SYSTEMS

Compound	Solvent system [a]		
	System A	System B	System C
	Rf value		
2,6-Dimethylnaphthalene	0.82	0.92	0.74
2,6-Dimethyl-3-naphthol	0.66	0.67	0.66
2,6-Dimethyl-3,4-naphthoquinone	0.71	0.60	0.43
6-Methyl-2-naphthalenemethanol	0.52	0.47	0.59
trans-3,4-Dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene	0.35	0.41	0.48
α-Naphthyl sulfate	0	0.41	0.20
β-Naphthyl-α-D-glucoside	0	0.29	0.15
α-Naphthyl-β-glucuronide	0	0.09	0.05

[a] System A, toluene:ethanol, 92:8 (v/v) in an unlined tank. System B, developed with toluene:ethyl acetate, 85:15 (v/v) to 15 cm in an unlined tank; then developed with ethanol:acetonitrile:acetic acid, 90:10:1 (v/v/v) to 8 cm in a filter paper-lined tank. System C, developed with toluene: acetone:acetonitrile, 80:10:10 (v/v/v) to 15 cm in a lined tank; then developed with n-butyl alcohol:water: ammonium hydroxide, 85:13:2 (v/v/v) to 8 cm in a lined tank.

within 24 hours much of the labeled DMN had been absorbed and metabolized by the fish. Thus, it was concluded that a 24-hour exposure of fish to the ^{14}C-2,6-DMN was suitable for the major experiment.

For the major experiment, the xenobiotics were dissolved in peanut oil and administered in doses of 0.2 ml/fish. The appropriate peanut oil solution was deposited into the stomach, via the mouth, using a stainless steel cannula. The experiment was conducted over a period of 9 days. On each of the first 6 days the fish were given the following: group 1, peanut oil only; group 2, 12 μg (0.09 μmol) of naphthalene (0.44 mg/kg body wt.); group 3, 9.7 μg (0.09 μmol) of p-cresol (0.33 mg/kg); group 4, 12 μg of naphthalene (0.44 mg/kg) and 9.7 μg of p-cresol (0.36 mg/kg). On day 7, no treatment took place. On day 8, each fish was given an oral dose of 0.2 ml of peanut oil containing 4.8 μCi of ^{14}C-2,6-DMN (0.35 mg/fish; average dose 12-15 mg/kg).

On day 9 the fish were sacrificed, and enlarged gall bladders were removed and frozen at -60°C.

Bile samples from four fish were pooled to yield three samples, in amounts ranging from 51 to 201 mg, for each of the four groups. Total bile for the four groups ranged from (mean ± S.D.) 96±52 mg to 134±59 mg.

The livers from the 48 starry flounder which received the ^{14}C-2,6-DMN were excised and frozen immediately after sacrifice. Four livers were combined in each case to provide a total of three pooled samples per group. Each pooled liver sample was divided into two portions. One portion was used for preparation of microsomes and assay for activity of benzo[a]pyrene monooxygenase (DePierre et al., 1975). The other portion was homogenized and extracted for HPLC analysis of metabolites of 2,6-DMN.

Analysis of 2,6-DMN Metabolites in Liver--

Metabolites of 2,6-DMN were extracted from the 12 pooled liver samples. The livers were weighed, minced, and homogenized in isopropyl alcohol:water (25:2, v/v) using three parts of solvent for one part of liver. Two 60-μl aliquots were removed to measure total radioactivity and radioactivity as unmetabolized 2,6-dimethylnaphthalene. Then, 100 μl of the 2,6-dimethyl[^{3}H]naphthalene-labeled rat urine extract (see sub-section below) were added as an internal standard, so as to estimate recoveries of metabolites. The homogenates were filtered through filter paper, and the residue on the paper was extracted three times with 8 ml portions of boiling methylene chloride:isopropyl alcohol:water (75:25:2, v/v/v). The residue was next extracted three times with 8 ml portions of boiling ethanol:ethyl acetate (50:50, v/v). The original filtrate and the extracts were combined and concentrated under a stream of nitrogen over an ice bath. When the extract volume reached less than 1 ml, 40 ml of anhydrous methanol were added and the concentration step was repeated. This solvent exchange was repeated, and the final methanol extract was concentrated to about 1.5 ml and used for HPLC analysis.

Tritiated metabolites of 2,6-DMN from rat urine--The procedures of Thomas et al. (1978), for naphthalene metabolites, were employed to assess the recoveries of ^{14}C-2,6-DMN metabolites from the liver and bile of the starry flounder. Accordingly, a 200 g male rat was injected intraperitoneally with 2,6-dimethyl[^{3}H]naphthalene dissolved in peanut oil. Urine was collected for 3-4 days following the injection. One week after the injection, the rat was reinjected with a second dose of the tritiated substrate. The urine samples containing the highest total radioactivity were combined prior to extraction of tritiated metabolites.

Our initial attempts to prepare the urine extracts consisted of saturating the urine with sodium chloride, followed by multiple extractions with ethyl acetate. We found this procedure yielded poor recoveries of total radioactivity. Much better results were obtained by applying the procedures of Horning et al. (1974). The urine was saturated with powdered ammonium carbonate and then extracted with ethyl acetate. The aqueous phase was carefully acidified to pH 1 and reextracted with ethyl acetate. The ethyl acetate fractions were combined, evaporated to near-dryness under nitrogen, and the residue taken up in a small volume of absolute methyl alcohol. This procedure allowed 60-80% recovery of the radioactivity in the urine samples. HPLC of the rat urine extracts indicated that they contained sufficient ^3H for liquid scintillation counting and also enough UV absorbing peaks for use in assessing the recoveries of ^{14}C-2,6-DMN and its metabolites in starry flounder tissues.

HPLC analysis of metabolites from liver--Separation of 2,6-DMN metabolites was accomplished by HPLC. Metabolites were eluted with a 60-min linear gradient beginning with 100% aqueous 10% monobasic potassium phosphate (5×10^{-4} M) and proceeding to 100% methanol. Fractions from the HPLC were collected at one-half minute intervals from zero to 70 minutes. Each fraction volume was 0.5 ml. Seven ml of scintillation fluid were added to each fraction, and the samples were counted for both ^{14}C and ^3H in a liquid scintillation spectrometer.

Separation and Identification of 2,6-DMN Metabolites from Bile--

Work on methods of analysis of biliary metabolites was directed at comparisons of procedures employing techniques with Amberlite XAD-2 and XAD-4 resins and preparative TLC. The procedures with XAD resins were patterned after those reported by Statham et al. (1976, 1978), while the TLC procedures included those reported by Roubal et al. (1977) as well as new procedures (described above). The methods were evaluated for optimum conditions for the recovery and separation of both conjugated and nonconjugated metabolites of 2,6-DMN from bile. Semi-quantitative analysis of recovery and evaluations of separations by the XAD-resin method and TLC method employed ^{14}C-labeled compounds in bile taken from the flounder in the initial small-scale experiment.

In the major experiment, labeled metabolites of the bile samples were separated by TLC. TLC system A was used to estimate the relative proportions of metabolites recovered as conjugated or nonconjugated products. Individual conjugated and nonconjugated metabolites were assayed using either TLC system B or system C. Labeled compounds were quantitated from resultant data of liquid scintillation counting of scraped TLC bands. Results of liquid scintillation counting are expressed as mole-% of total metabolites.

The glucuronides, i.e., compounds in a TLC band having an Rf corresponding to that of the standard α-naphthyl-β-glucuronide, were recovered from the silica gel by extraction with methanol. The glucuronides were treated with limpet β-glucuronidase (2,140 Fishman units) in 0.1 M citrate-phosphate buffer, pH 4.0, at 37^o for 3 hr (Dodgson et al., 1953). Blanks contained either the β-glucuronidase-inhibitor D-saccharo-1,4-lactone (Levvy, 1952) or heat-denatured β-glucuronidase. Following hydrolysis, the reaction mixtures were acidified with 0.1 ml of 6M-HCl, and the products were extracted into peroxide-free ethyl ether. After drying with Na_2SO_4 and concentration under nitrogen, the extracts were analyzed by TLC. The efficacy of the β-glucuronidase action was verified by the hydrolysis of α-naphthyl-β-glucuronide to yield α-naphthol (detected by spraying with Fast Blue Salt B; Boyland and Solomon, 1956), and by autoradiography of TLC plates using Kodak X-Omat R film. More than 90% of the material in the glucuronide fraction was hydrolyzed by β-glucuronidase.

The glucosides (TLC section with Rf corresponding to β-naphthyl-α-glucoside) were extracted from silica gel with methanol and hydrolyzed at pH 6.8 (citrate-phosphate) first with α-glucosidase (13 units) for 3 hr at 37°C, then with β-glucosidase (23 units) for 2 hr at 37°. The blanks were run at pH 10, which inhibited the glucosidases. The products of the hydrolysis were recovered and analyzed as described for the glucuronides. More than 50% of the material in the glucoside fraction was hydrolyzed by mixed α- and β-glucosidases.

Statistics--

Statistical analyses involving two-sided comparisons of treatment groups versus the control group (Dunnett, 1955; Dunnett, 1964) were performed on data from replicate samples. Student's t-test was used to compare sizes of fish.

Results

2,6-DMN Metabolites from Livers--

Tables 27 and 28 present the qualitative results of the HPLC analyses of ^{14}C-2,6-DMN metabolites extracted from the starry flounder liver samples. For these analyses, five compounds were utilized as external standards: 2,6-DMN, trans-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene, 2,6-dimethyl-3,4-naphthoquinone (containing a persistent contaminant), 2,6-dimethyl-3-naphthol, and 6-methyl-2-naphthalenemethanol. Table 27 presents the distributions of total metabolites and residual substrate from the four experimental groups. The data in Table 27 are further resolved and presented in Table 28.

The results presented in Table 28 indicate three significant peaks for metabolites in the liver samples from the control groups, the p-cresol-exposed groups and the naphthalene-exposed group. The liver samples from the group of fish exposed to both p-cresol and naphthalene exhibited five significant peaks of radioactivity which we attribute to metabolites of 2,6-dimethylnaphthalene. Figure 21 is a graphical representation of the metabolite profile for the livers of the group of fish exposed to both p-cresol and naphthalene. The graph also indicates the retention times of the external standards.

2,6-DMN Metabolites from Bile--

The results of experiments with XAD-2 and XAD-4 resins showed no benefit of one resin over the other in analyses of biliary metabolites. Reference standards of 2,6-DMN, 2,6-dimethyl-3-naphthol, and the trans-3,4-dihydrodiol of 2,6-dimethylnaphthalene were retained on the resins; the latter two derivatives elute with methanol and the parent compound elutes with acetone. In another experiment where bile containing ^{14}C-labeled metabolites was passed through XAD-2 resin, the initial water eluate removed 2.5% radioactive substances in relation to those substances removed with methanol. The water eluate is normally discarded according to procedures of Statham et al. (1976, 1978), so metabolites are lost by the resin technique. Also, losses can occur during evaporation of solvents, e.g., when 50 ml of a methanol extract are concentrated.

Preparative TLC procedures offered advantages over the procedures with XAD resins by allowing less sample handling and providing separations in a much shorter time. Preparative TLC and liquid scintillation spectrometry of

TABLE 27. PERCENT OF ^{14}C 2,6-DIMETHYLNAPHTHALENE METABOLIZED IN STARRY FLOUNDER LIVERS AS DETERMINED BY HPLC ANALYSIS [a]

	Control group	Exposure group		
		p-Cresol	Naphthalene	p-Cresol and naphthalene
% as Metabolites	23.5	26.5	17.0	31.0
% as Substrate	76.4	73.5	82.9	68.9

[a] Percentages calculated on the basis of ^{14}C radioactivity recovered from HPLC as parent compound or as metabolites. Livers of 12 fish per group were pooled and extracted, the extracts were concentrated to 0.75-1.5 ml, and 0.1-0.2 ml of the concentrates were analyzed by HPLC.

TABLE 28. ANALYSIS OF ^{14}C 2,6-DIMETHYLNAPHTHALENE METABOLITES BY HPLC OF LIVER EXTRACTS FROM STARRY FLOUNDER FED p-CRESOL AND NAPHTHALENE, SEPARATELY AND TOGETHER: RADIOACTIVITY IN HPLC FRACTIONS

Retention time	Control group	Exposure group		
		p-Cresol	Naphthalene	p-Cresol and naphthalene
min	Dpm			
12.5 - 16.5	135	107	164	49
16.5 - 20.0	nil	nil	nil	171
20.0 - 25.0	551	532	1135	1110
25.0 - 30.0	nil	nil	nil	173
30.0 - 35.0	nil	nil	nil	167
35.0 - 40.0 [a]	16	57	91	97
40.0 - 50.0	nil	nil	nil	nil
50.0 - 70.0 [b]	2277	1934	6749	3920

[a] Corresponds to trans-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene

[b] Corresponds to 2,6-dimethylnaphthalene

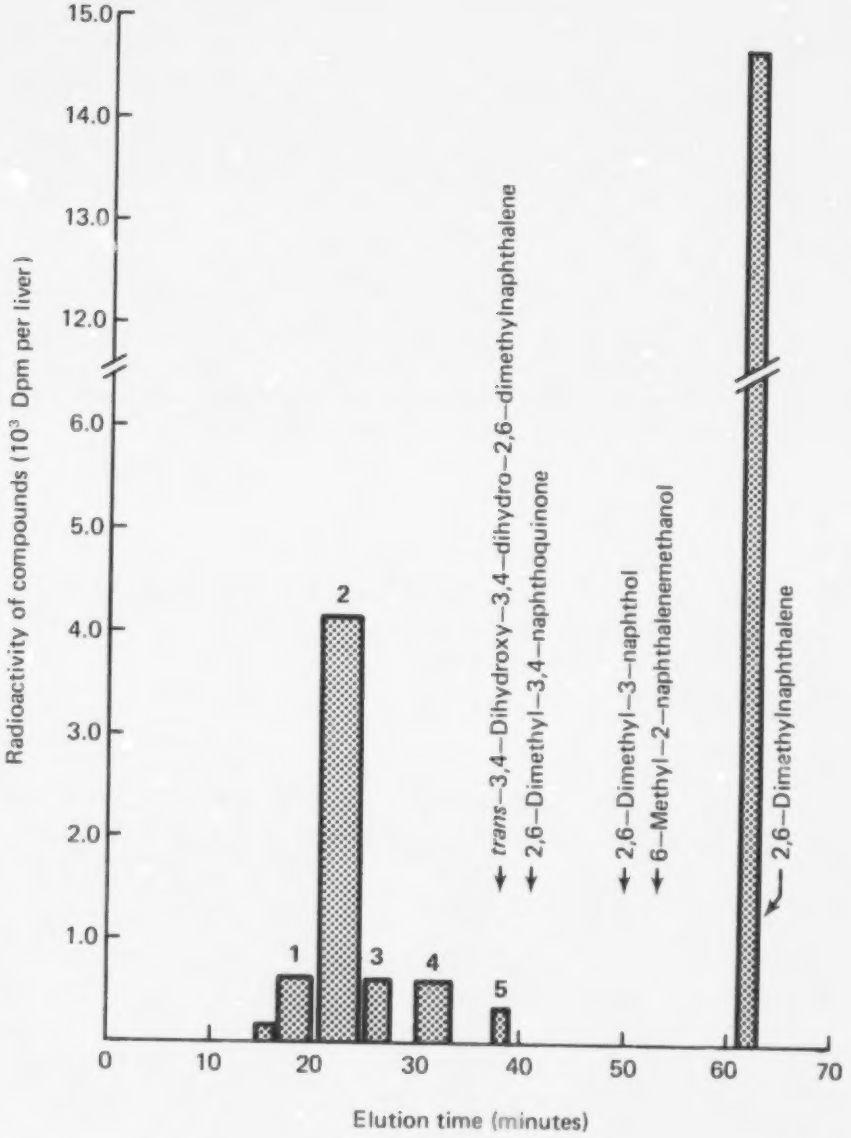


FIGURE 21. HPLC profile of metabolites of 2,6-dimethylnaphthalene-4-^{14}C from livers of starry flounder exposed to naphthalene and p-cresol. Elutions of reference standards are indicated by arrows.

TLC fractions (bands) were used to assess the optimum formation of biliary metabolites from fish in the short-term exposure experiment discussed previously. The biliary metabolites appeared to reach maximum concentrations between 12 and 48 hr. Also, analyses showed that the metabolites were nearly all conjugated derivatives.

Other TLC analyses of biliary metabolites from 12- and 48-hr samples, using the forementioned reference standards, showed that the conjugated metabolites corresponded to standards for naphthylglucoside and naphthylglucuronide.

The concentrations of metabolites of 2,6-DMN found in the four groups of starry flounder in the major experiment are presented in Table 29. Three nonconjugated products were isolated and quantitated: 2,6-dimethyl-3-naphthol; 2,6-dimethyl-3,4-naphthoquinone; and 6-methyl-2-naphthalenemethanol. The method employed (TLC system B) did not resolve trans-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene and aryl sulfate conjugates. Conjugated metabolites of 2,6-DMN constituted 54.6 mole-% to 77 mole-% of metabolites.

Comparisons of the controls with the xenobiotic-exposed fish indicated no statistically significant differences ($P>0.05$) between the amounts of the glucoside fraction (Table 29). Enzymatic hydrolysis of the glucoside fraction was incomplete; therefore, this fraction was not analyzed further. The concentrations of glucuronides in groups 2 and 3 are not significantly different from those in group 1. However, the concentration of glucuronides in group 4 (naphthalene and p-cresol, 19.9 mole-%) is significantly lower ($P\leq 0.05$) than that in group 1 (controls, 34.8 mole-%).

When the glucuronides of 2,6-DMN metabolites were hydrolyzed, more than 98% of the radioactivity was accounted for by two products: 6-methyl-2-naphthalenemethanol (alcohol) and trans-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene (dihydrodiol). The results are presented in Table 30. There was a significant reduction ($P\leq 0.01$) in the concentrations of the alcohol metabolite in groups 2, 3, and 4 relative to group 1. Correspondingly, there was an increase in the amount of the glucuronide of the dihydrodiol. This increase, relative to the control group, was more significant for groups 2 and 3 ($P\leq 0.01$) than it was for group 4 ($P\leq 0.05$).

Measurements of radioactivity in aliquots of bile samples showed variations of 0.26% to 0.72% of the administered dose present in the bile after 24 hr. There were no statistical differences in the percentages of dose present in bile between treatment groups and controls.

Microsomal Benzo[a]pyrene Monooxygenase--

Microsomes, prepared from the 12 pooled liver samples from each group, were assayed for benzo[a]pyrene monooxygenase activity. The low limit of measurement of the monooxygenase activity in our assay system is 0.2 nmol of products formed per mg of microsomal protein for a 20-min incubation (Gruger et al., 1977a, 1977b). In all enzyme assays, the microsomes possessed the same low activities of ≤ 0.2 nmol/mg; therefore, no effect on the monooxygenase activity could be attributed to the xenobiotics fed to the fish.

Discussion

The experiments were designed to ascertain whether exposure of starry flounder to naphthalene and/or p-cresol alters the *in vivo* metabolism of 2,6-DMN. A detailed description of the metabolism of this alkylated naphthalene in fish has not been reported previous to the present experiments (Gruger et al., 1981).

The results demonstrate that these flatfish can oxidize 2,6-DMN to at least four products: a naphthol, a quinone, a dihydrodiol and an alcohol. The bile contained both nonconjugated and conjugated metabolites, with the conjugated derivatives predominating in all four groups. The most important feature of the data on the nonconjugated metabolites is the finding that fish metabolize 2,6-DMN by either aromatic-ring or side-chain oxidation. Another methylated polycyclic aromatic hydrocarbon, 7,12-dimethylbenz(a)anthracene, has been shown to be metabolized by rats by either aromatic-ring or side-chain oxidation (Jellinck and Goudy, 1967).

In all four groups of fish (Table 29), the glucoside fraction of 2,6-DMN metabolites constituted the largest class of identified conjugates (range = 34.7 to 42.5 mole-%). In a closely comparable experiment (Varanasi et al., 1979), it was found that in 24 hrs, starry flounder, which were force-fed ^{3}H-naphthalene, yielded sulfate/glucoside conjugates as 1.4% of total biliary metabolites of naphthalene. Comparison of these two experiments indicated that the presence of the alkyl substituents on the naphthalene nucleus leads to increased proportions of biliary glucoside conjugates.

The data of Table 29, indicating that simultaneous exposure to naphthalene and p-cresol decreased the proportion of 2,6-DMN metabolites recovered as biliary glucuronides, suggest an interactive effect; i.e., the two xenocompounds act together to elicit the change in composition of 2,6-DMN metabolites. This conclusion, however, must be tempered by two facts. First, the total molar xenobiotic dose in group 4 (0.18 μmole/fish) is twice that in either group 2 or group 3 (0.09 μmole/fish). The effect observed in group 4 might have occurred if either naphthalene or p-cresol had been tested at a dose of 0.18 μmole/fish; a more complicated experimental protocol would be needed to resolve this question. The second fact which must be considered is that the reduction in the glucuronide fraction of group 4 was accompanied by an increase in the 2,6-DMN metabolites recovered by TLC as "unidentified migrating fraction" (Table 29). Therefore, although there was a statistically significant reduction ($P \leq 0.05$) in the glucuronide fraction for group 4, the nature of the metabolic changes is presently unknown.

The results (Table 30) support the conclusion that exposure to naphthalene alone had a greater effect on the metabolism of 2,6-DMN than did exposure to p-cresol alone. This conclusion is reasonable in view of the physical and chemical properties of the two compounds. p-Cresol is both more volatile and more soluble in water than is naphthalene. Thus, p-cresol can probably be excreted more effectively. Furthermore, p-cresol could undergo direct conjugation at its hydroxy group without the need for oxidative metabolism; this may also favor rapid excretion by the fish.

TABLE 29. 2,6-DIMETHYLNAPHTHALENE-4-^{14}C METABOLITES IN BILE OF STARRY FLOUNDER EXPOSED TO NAPHTHALENE AND P-CRESOL[a]

Metabolite fraction	Controls (Group 1)	Exposure group Naphthalene (Group 2)	p-Cresol (Group 3)	Naphthalene and p-Cresol (Group 4)
	- - - -Mol-% in total metabolites[b] - - - - - - - - -			
2,6-Dimethyl-3-naphthol[b]	4.4 ± 4.9	5.4 ± 2.9	5.4 ± 3.1	7.1 ± 4.7
2,6-Dimethyl-3,4-naphthoquinone[b]	0.60± 0.53	1.3 ± 0.64	0.87± 0.21	3.5 ± 4.8
6-Methyl-2-naphthalene-methanol	1.2 ± 0.21	4.1 ± 2.0	2.6 ± 0.62	2.7 ± 1.5
trans-3,4-Dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene and sulfates	1.9 ± 0.87	5.1 ± 0.62	2.0 ± 0.60	3.5 ± 0.40
Glucosides[b]	42.5 ± 4.7	39.1 ± 2.3	37.1 ± 6.4	34.7 ± 4.2
Glucuronides[b]	34.8 ± 4.4	26.8 ± 6.4	35.5 ± 7.4	19.9 ± 3.8[c]
Unidentified migrating fractions	6.8 ± 2.5	6.4 ± 4.9	8.3 ± 2.9	19.0 ± 2.4
Unidentified, R_f= 0	7.8 ± 1.9	11.7 ± 3.8	8.2 ± 0.90	9.5 ± 1.9
Total	100	99.9	100	99.9

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[a] Biliary metabolites 24 hours after a force-fed dose of ^{14}C-2,6-dimethylnaphthalene (12-15 mg/kg, 4.8 μCi/fish, 2.1 mCi/mmole). Mean values ± S.D. (n = 3); pooled samples from 4 fish; 12 fish/group.

[b] Metabolites separated and identified by TLC System B (cf., Table 26).

[c] Significantly different ($P \leq 0.05$) from control group.

TABLE 30. METABOLITES OF 2,6-DIMETHYLNAPHTHALENE-4-^{14}C FROM ENZYMATIC HYDROLYSIS OF THE GLUCURONIDE FRACTION OF STARRY FLOUNDER BILE SAMPLES [a]

Group Number	Group Treatment	6-Methyl-2-naphthalenemethanol	t-3,4-Dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene	Ratio 6-Me-2-naphthalenemethanol / Dihydrodiol
		- - - - - - - - - - - - - - Mole %	- - - - - - -	
1	Control	31.4 ± 4.0	3.43 ± 0.43	9.15
2	Naphthalene	10.4 ± 2.5[b]	16.4 ± 3.9[b]	0.63
3	p-Cresol	19.5 ± 4.1[b]	15.9 ± 3.3[b]	1.23
4	Naphthalene and p-cresol	8.25 ± 2.1[b]	11.7 ± 1.8[c]	0.71

[a] Biliary metabolites 24 hours after a force-fed dose of ^{14}C-2,6-DMN (4.8 μCi/fish, 2.1 mCi/mmole). Mean values ± S.D. ($n = 3$); pooled bile samples from 4 fish; 12 fish/group.

[b] Significantly different ($P \leq 0.01$) from control group.

[c] Significantly different ($P \leq 0.05$) from control group.

The glucuronide fraction of 2,6-DMN metabolites from each group of fish was subjected to further analysis (Table 30). Only two products resulted when the glucuronides were hydrolyzed with β-glucuronidase. One of these products, the dihydrodiol, represents hydroxylation of the aromatic rings of 2,6-DMN; the other, the alcohol, is produced by oxidation of one of the methyl substituents. The most intriguing aspect of the data (Table 30) is the change in the relative proportion of the dihydrodiol and the alcohol following exposure to either xenobiotic or to the mixture of the two pollutants. In each exposure group, there was a shift from methyl-group oxidation to aromatic-ring oxidation (hydroxylation). This alteration was most pronounced for the fish of group 2 (exposure to naphthalene only). An analogous result has been reported in rats where it was demonstrated that exposure to polycyclic aromatic hydrocarbons altered hepatic microsomal metabolism of 7,12-dimethylbenz(a)anthracene from side-chain oxidation to ring hydroxylation (Jellinck and Goudy, 1967).

It is well established that the oxidation of naphthalene to dihydrodiols is catalyzed by aryl hydrocarbon monooxygenases (Jerina et al., 1970); however, far less is known about the enzymes responsible for oxidation of alkyl side chains of methylated naphthalenes (Kaubisch et al., 1972). Thus, it is not possible to interpret our results in terms of specific changes in the levels or specificities of identified enzymes. Nevertheless, the principal finding is clear: in starry flounder, previous exposure to an aromatic compound alters the nature of the metabolism of 2,6-DMN *in vivo*.

Our demonstration that exposure to naphthalene results in an increased proportion of dihydrodiol formation from 2,6-DMN could mean that exposure to similar aromatic compounds may lead to a different formation of products, and thus alter the potential toxicity of products, upon subsequent exposure to other aromatic chemicals. This type of interactive effect of two (or more) chemicals does not necessarily require the simultaneous presence of both (or all) chemicals. The present results have shown that exposure of a demersal fish to xenobiotics, especially repeated exposures to aromatic hydrocarbons, may affect the subsequent metabolism and potential toxicity of aromatic xenobiotics.

Summary

Juvenile starry flounder were force-fed naphthalene, p-cresol, or a mixture of naphthalene and p-cresol in daily doses of 0.3-0.4 mg/kg body weight, for six consecutive days. On the eighth day, each fish was force-fed a dose of 12-15 mg/kg of ^{14}C-2,6-dimethylnaphthalene (2,6-DMN). Twenty-four hours later, the fish were killed and ^{14}C-labeled metabolites in the bile were isolated and identified by TLC.

Most of the biliary metabolites were recovered as conjugates, principally as glucosides and glucuronides. Analyses of the nonconjugated metabolites and compounds resulting from enzymatic hydrolysis of the conjugate metabolites provided identification of four metabolites of 2,6-DMN: 2,6-dimethyl-3-naphthol; 2,6-dimethyl-3,4-naphthoquinone; *trans*-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene (dihydrodiol); and 6-methyl-2-naphthalenemethanol (alcohol). Enzymatic hydrolysis of the glucuronides yielded two compounds:

the alcohol, representing metabolism at a methyl substituent, and the dihydrodiol, representing oxidation of an aromatic ring. Exposure to naphthalene and/or p-cresol led to a significant reduction ($P \leq 0.05$) in the proportion of the alcohol product and a corresponding increase in the proportion of the dihydrodiol. This perturbation, which favors the formation of potentially damaging epoxides, may alter the nature of toxicological effects of such aromatic hydrocarbons.

EFFECTS OF NAPHTHALENE AND *P*-CRESOL ON *IN VIVO* SYNTHESIS OF LIPIDS IN STARRY FLOUNDER

The purpose of the experiment was to study the effect of xenobiotics on the *in vivo* lipid biosynthesis in starry flounder.

Method

Exposure Protocol --

The xenobiotics were administered by intraperitoneal injections. Each fish of the control group was injected with the solvent (80% aqueous ethanol; 1 ml/kg). Each fish of the naphthalene group received 5 mg/kg of naphthalene (5 mg/kg in 80% ethanol) per injection. Each fish of the p-cresol group received 5 mg/kg of p-cresol (5 mg/kg in 80% ethanol) per injection. Each fish of the fourth group received 5 mg/kg of naphthalene and 5 mg/kg of p-cresol per injection.

Immature starry flounder (1 1/2-2 yr old) were used for this experiment. There were 20 fish for each of the three xenobiotic-treatment groups and 24 fish for the control group. On the first day all fish were injected with the appropriate xenobiotic solution. Twenty-four hr later the injections were repeated. Forty-eight hr after the initial injection, four fish from the control group were sacrificed and samples were taken for histological examinations. At the same time, four fish from each group were sacrificed, and tissue samples were taken for analyses of uptake of xenobiotics and activities of liver microsomal 2,6-DMN monooxygenase.

Four fish from each of the three treatment groups received labeled xenobiotics [1-^{14}C-naphthalene and/or p-cresol($^{14}CH_3$), each with a specific radioactivity of 10 μCi/mg]. Forty-eight hr after the initial injection, four fish from each xenobiotic-treatment group were sacrificed, and liver, gill, intestine, and muscle samples were taken. All of these tissues were assayed for distribution of ^{14}C. Liver homogenates were prepared and the microsomes were assayed for 2,6-DMN monooxygenase activity.

Forty-eight hr after the initial injections, each of the remaining fish (16 per group; total of 64) was given a single intraperitoneal injection of labeled lipid precursors [1-^{14}C-acetate, 59 mCi/mmole; (^{3}H-9,10)oleate, 6.4 Ci/mmole; both dissolved together in 0.9% aqueous sodium chloride made pH 9.5 with KOH]. Four fish from each group were sacrificed at intervals of 1, 2, 4, and 10 hr after administration of the acetate and oleate. The fish were rapidly frozen on dry ice and held frozen until samples could be taken and analyzed. Mean lengths and weights of all 80 fish are presented in Table 31.

Analyses --

Monooxygenase activity of hepatic microsomes was assayed using 2,6-DMN as the substrate (Schnell et al., 1980). Distribution of radioactivity due to 1-^{14}C-naphthalene and/or p-cresol($^{14}CH_3$) was measured by digestion of tissues in 4 N NaOH and extraction into n-hexane (Collier, 1978; Varanasi et al., 1978).

Liver lipids were extracted by the procedure of Hanson and Olley (1963). The method of Hornstein et al.(1967) was used to separate lipid classes. TLC separations of phospholipids were performed on silica gel using chloroform:methanol:water (65:25:4, v/v/v) according to Wagner et al.(1961). Phosphorus content of the total phospholipids, phosphatidylethanolamines, and phosphatidylcholines was determined by the method of Bartlett (1959) as modified by Parker and Peterson (1965). Weights of extracted total lipids, fractions of neutral lipids (with free fatty acids), and phospholipids were determined by microgravimetry.

Results

Histological examinations of four control fish indicated no morphological abnormalities; thus, the starry flounder were healthy specimens. Distributions of ^{14}C-labeled compounds in fish tissues following injections with 1-^{14}C-naphthalene and/or p-cresol($^{14}CH_3$) are presented in Table 32. All tissues examined showed significant radioactivity indicating that the xenobiotics were distributed throughout the bodies of the fish. In the ^{14}C-naphthalene group, the radioactivity in the alkaline phase of the extracts exceeded that in the hexane layer; this was seen in all four tissues assayed. These results indicate that extensive metabolism of the ^{14}C-naphthalene had taken place.

Monooxygenase activities of liver microsomes toward 2,6-DMN are presented in Table 33. These data indicate that the naphthalene-treated group had significantly higher monooxygenase activity than did the control group. Enzyme activities for the p-cresol-treated group and the group of fish which received both xenobiotics were not significantly different from the controls.

Only the 4-hr and 10-hr liver samples were evaluated for lipid content and lipid biosynthesis. Lipid contents of the livers are summarized in Table 34; a range of 2.9 to 6.8 mg-% was found.

Data for the 4-hr incorporation of the lipid precursors into neutral lipids and phospholipids are presented in Table 35. Neutral lipids were more highly labeled than were phospholipids, whether the precursor was tritiated oleate or ^{14}C-labeled acetate. There were no

TABLE 31. STARRY FLOUNDER USED FOR STUDY OF LIPIDS

Treatment group	Sampling time	Fish weight	Fish length
	hr	g	mm
Control	1	92 ± 25	173 ± 10
	2	89 ± 81	159 ± 41
	4	126 ± 58	184 ± 29
	10	87 ± 54	166 ± 26
	0 [a]	130 ± 58	230 ± 37
Naphthalene	1	114 ± 61	174 ± 31
	2	148 ± 43	184 ± 17
	4	88 ± 33	160 ± 15
	10	92 ± 40	157 ± 25
	0 [a]	122 ± 30	231 ± 16
p-Cresol	1	82 ± 74	153 ± 42
	2	112 ± 69	176 ± 32
	4	74 ± 38	158 ± 29
	10	92 ± 35	171 ± 21
	0 [a]	120 ± 12	231 ± 22
Naphthalene and p-cresol	1	62 ± 13	151 ± 6
	2	135 ± 84	178 ± 38
	4	88 ± 55	166 ± 29
	10	100 ± 85	163 ± 34
	0 [a]	139 ± 42	238 ± 14

[a] Used for enzyme assays and uptake analyses. Mean ± S.D. (n = 4).

TABLE 32. SOLVENT DISTRIBUTIONS OF CARBON-14 LABELED SUBSTANCES FROM TISSUES OF STARRY FLOUNDER AT 24 HOURS AFTER FINAL INTRAPERITONEAL INJECTIONS OF 1-^{14}C-NAPHTHALENE AND P-CRESOL($^{14}CH_3$)

Treatment group [a]	Solvent	Radioactivity in tissues [b]			
		Liver	Gill	Intestine	Muscle
		- - - - - - - - - - - - Dpm/g wet tissue - - - - - - - - - -			
Naphthalene	Hexane	3,210	570	1,190	240
	Aqueous NaOH	7,990	1,180	3,180	630
p-Cresol	Hexane	310	20	240	100
	Aqueous NaOH	27,090	27,800	56,000	3,630
Naphthalene and p-cresol	Hexane	6,350	840	1,490	540
	Aqueous NaOH	19,870	13,800	47,000	2,930

[a] Treatments were made by i.p. injections on each of two consecutive days of single doses of naphthalene and/or p-cresol at 5 mg/kg body weight. Samples were taken at 24 hours after the second injections. Specific activity of the injection fluids were 50 μCi/5mg/ml of each compound in 80% ethanol.

[b] Values are the averages for four fish. Distribution is between 4N-NaOH (after digestion) and n-hexane, following extractions according to Varanasi et al. (1978).

statistically significant differences among the four experimental groups. The incorporation of tritiated oleate into sterol esters and triglycerides is summarized in Table 36. Those data indicate a significant decrease in incorporation of (^{3}H-9,10)oleate into triglycerides (at 4 hr) as a result of prior exposure of the fish to p-cresol and/or naphthalene. Differences between the xenobiotic-treatment groups and the control group were not statistically significant in incorporation of ^{3}H-oleate into sterol esters.

The incorporation of ^{14}C-acetate into free fatty acids, triglycerides, and sterol esters is presented in Table 37. Significant decreases in acetate incorporation into fatty acids were seen due to the prior treatment with naphthalene ($P < 0.05$) or with both naphthalene and p-cresol ($P < 0.01$). Treatment with p-cresol alone did not produce a statistically significant change in fatty acid synthesis from acetate.

Table 38 is a summary of the results obtained from incorporation of both ^{3}H-oleate and ^{14}C-acetate into total phospholipids, phosphatidylethanolamines, and phosphatidylcholines. There was greater radioactivity in all products at 4 hr than at 10 hr, but there were no significant differences among the four groups of fish.

Discussion

The results presented for the distribution of 1-^{14}C-naphthalene and p-cresol($^{14}CH_3$) indicate that there was significant uptake of the xenobiotics during the course of this experiment. Also, it was evident that ^{14}C-naphthalene was extensively metabolized by the starry flounder. The data of Table 33 demonstrates that the fish livers possess highly active mixed-function oxidases capable of metabolizing PAH's (Schnell et al., 1980). The elevation of mixed-function oxidase activity in the naphthalene-treated group compared to the control group suggests that some induction of monooxygenase activity had taken place during the 48-hr treatment with naphthalene. Thus, it is clear that the xenobiotics had ample opportunity to effect metabolic changes in the experimental animals.

Two significant findings resulted from the investigation of effects of naphthalene and p-cresol on in vivo lipid biosynthesis. At 4 hr, there was a demonstrable decrease in triglyceride synthesis from ^{3}H-oleate due to treatment with naphthalene, p-cresol, or both organic perturbants. Precisely how these chemicals exert this effect is uncertain. A detailed study of the enzymatic steps involved might be required to ascertain the location of this change in the overall pathways of triglyceride biosynthesis.

The second notable finding is that naphthalene diminished the biosynthesis of fatty acids from the ^{14}C-acetate precursor. p-Cresol treatment alone did not elicit the same effect. It would be interesting to ascertain whether other PAH's could produce the same result as naphthalene. Naphthalene is certainly less polar than p-cresol and, thus, may have greater access to the cellular organelles and processes which are responsible for the control of lipid biosynthesis from a two-carbon precursor of fatty acids.

Summary

Starry flounder exposed for 48 hours to naphthalene, p-cresol, or a 50-50 mixture of the two compounds, by intraperitoneal injections, were found to decrease the incorporation of tritiated oleic acid into triglycerides of hepatic lipids. Only naphthalene was found to decrease the incorporation of 1-^{14}C-acetate into fatty acids. No significant effects were demonstrated with phospholipid biosynthesis. Naphthalene-treated flounder exhibited enhanced activity of hepatic microsomal aryl hydrocarbon (2,6-dimethylnaphthalene) monooxygenase. The data suggest that measurements of incorporation of fatty acids into triglycerides in liver lipids may be a sensitive indicator of xenobiotic exposures.

TABLE 33. ACTIVITY OF HEPATIC 2,6-DIMETHYLNAPHTHALENE MONOOXYGENASE FOR STARRY FLOUNDER TREATED WITH NAPHTHALENE AND p-CRESOL BY INTRAPERITONEAL INJECTIONS, 24 HOURS AFTER TWO INJECTIONS 24 HOURS APART

Treatment group	Monooxygenase activity[a]	Percent of control
	pmole/mg protein/min	
Control	15.2 ± 16.4	100
Naphthalene	50.7 ± 28.6[b]	334
p-Cresol	22.2 ± 5.8	146
Naphthalene and p-cresol	26.7 ± 11.7	176

[a] Hepatic microsomes analyzed according to the procedures of Schnell et al. (1980).

[b] $P \leq 0.05$ relative to control group.

TABLE 34. LIPID CONTENT OF STARRY FLOUNDER LIVERS USED TO DETERMINE THE INCORPORATION OF LIPID PRECURSORS AT 4-HOUR AND 10-HOUR INTERVALS

Treatment group	Time interval	Fish body weight	Liver weight		Lipid content
			Total	Pooled	
	hr	g	g	g	mg-%
Control	4	191	0.796	1.428	4.28
	4	60	0.632		
	4	100	0.462	1.712	6.75
	4	155	1.250		
	10	165	1.250	1.449	6.46
	10	54	0.199		
	10	84	0.784	1.075	5.98
	10	46	0.291		
Naphthalene	4	58	0.325	1.344	5.01
	4	119	1.019		
	4	99	0.987	1.609	5.06
	4	75	0.622		
	10	114	0.697	1.254	4.84
	10	69	0.557		
	10	50	0.336	1.306	4.87
	10	137	0.970		

(continued)

TABLE 34 (continued)

Treatment group	Time interval	Fish body weight	Liver weight		Lipid content
			Total	Pooled	
	hr	g	g	g	mg-%
p-Cresol	4	46	0.332	1.419	3.82
	4	120	1.087		
	4	38	0.216	0.903	3.57
	4	91	0.687		
	10	71	0.472	1.639	2.91
	10	140	1.167		
	10	94	0.838	1.309	3.86
	10	63	0.471		
Naphthalene and p-cresol	4	83	0.948	1.675	5.15
	4	57	0.727		
	4	166	1.198	1.451	4.74
	4	43	0.253		
	10	55	0.810	1.332	5.68
	10	60	0.522		
	10	227	1.934	2.417	4.75
	10	57	0.483		

TABLE 35. FOUR-HOUR INCORPORATION OF (^{3}H-9,10)OLEATE AND 1-^{14}C-ACETATE INTO LIPIDS OF LIVERS FROM STARRY FLOUNDER TREATED WITH NAPHTHALENE AND P-CRESOL [a]

Treatment group	Specific activity in liver			
	Neutral lipid fraction [b]		Phospholipid fraction	
	^{3}H-labeled	^{14}C-labeled	^{3}H-labeled	^{14}C-labeled
	- - - - - - - - - - - - - - - picomoles/g liver - - - - - - - - - - - -			
Control	31.4 ± 9.0	1,870 ± 1,390	5.2 ± 1.8	179 ± 81
Naphthalene	25.8 ± 11.1	2,080 ± 686	4.8 ± 1.6	204 ± 22
p-Cresol	29.0 ± 5.4	1,760 ± 212	6.3 ± 0.3	152 ± 37
Naphthalene and p-cresol	17.0 ± 8.1	1,340 ± 584	3.9 ± 1.7	118 ± 11

[a] Specific activity of lipid precursors: (^{3}H-9,10)oleate, 6.4 Ci/mmole; 1-^{14}C-acetate, 59.0 mCi/mmole.

[b] Includes free fatty acids.

TABLE 36. INCORPORATION OF (^{3}H-9,10)OLEATE INTO STEROL ESTERS AND TRIGLYCERIDES OF LIVER LIPIDS FROM STARRY FLOUNDER TREATED WITH NAPHTHALENE AND P-CRESOL

Treatment	Time of incorporation	Radioactivity in lipids			
		Sterol esters		Triglycerides	
	hr	Dpm/mg total lipids	Dpm/g liver	Dpm/mg total lipids	Dpm/g liver
Control	4	192 ± 95	9,776 ± 1,901	442 ± 11	24,300 ± 7,142
	10	51 ± 28	3,130 ± 1,568	164 ± 165	9,944 ± 9,685
Naphthalene	4	61 ± 27	3,050 ± 1,324	78 ± 36[a]	3,892 ± 1,785[b]
	10	98 ± 3	4,759 ± 168	138 ± 74	6,716 ± 3,647
p-Cresol	4	154 ± 28	5,700 ± 1,316	166 ± 30[a]	6,186 ± 1,405[b]
	10	136 ± 60	4,398 ± 1,102	97 ± 22	3,224 ± 94
Naphthalene and p-cresol	4	70 ± 41	3,540 ± 2,244	85 ± 64[a]	4,290 ± 3,392[b]
	10	52 ± 21	2,617 ± 769	203 ± 40	10,716 ± 3,375

[a] $P \leq 0.01$ compared to controls at 4 hr (n = 2).

[b] $P \leq 0.05$ compared to controls at 4 hr (n = 2).

TABLE 37. INCORPORATION OF $1\text{-}^{14}C$-ACETATE INTO FREE FATTY ACIDS, TRIGLYCERIDES, AND STEROL ESTERS OF LIVER LIPIDS FROM STARRY FLOUNDER TREATED WITH NAPHTHALENE AND P-CRESOL

Treatment group	Time of incorporation	Radioactivity in lipids					
		Free fatty acids		Triglycerides		Sterol esters	
	hr	Dpm/mg total lipids	Dpm/g liver	Dpm/mg total lipids	Dpm/g liver	Dpm/mg total lipids	Dpm/g liver
Control	4	1,383 ± 130	75,150±16,990	180 ± 34	6,260±3,779	731 ± 153	41,650±21,190
	10	445 ± 358	27,080±20,770	59 ± 63	3,536±3,688	188 ± 176	11,400±10,320
Naphthalene	4	677 ± 157[a]	34,100± 8,160[a]	35 ± 3	1,768± 162	324 ± 271	16,370±13,740
	10	635 ± 48	30,820± 2,475	79 ± 43	3,843±2,077	390 ± 77	18,920± 3,825
p-Cresol	4	1,100 ± 156	40,800± 7,700	67 ± 66	2,529±2,584	710 ± 199	26,080± 6,081
	10	948 ± 49	31,930± 4,707	29 ± 5	988± 370	483 ± 204	17,040±10,140
Naphthalene and p-cresol	4	288 ± 70[b]	14,380± 4,282[b]	15 ± 16	784± 834	466 ± 446	23,700±23,420
	10	426 ± 36	22,120± 905	44 ± 4	2,305± 481	274 ± 96	14,610± 6,828

[a] $P \leq 0.05$ compared to controls at 4 hr (n = 2).

[b] $P \leq 0.01$ compared to controls at 4 hr (n = 2).

TABLE 38. INCORPORATION OF (^{3}H-9,10)OLEATE AND 1-^{14}C-ACETATE INTO PHOSPHOLIPIDS OF LIVER LIPIDS FROM STARRY FLOUNDER TREATED WITH NAPHTHALENE AND P-CRESOL [a]

Treatment	Time of incorporation	Radioactivity in phospholipids [b]					
		Total phospholipids		Phosphatidyl-ethanolamines		Phosphatidyl-cholines	
	hr	$\frac{^{3}\text{H-dpm}}{\mu\text{g Pi}}$	$\frac{^{14}\text{C-dpm}}{\mu\text{g Pi}}$	$\frac{^{3}\text{H-dpm}}{\mu\text{g Pi}}$	$\frac{^{14}\text{C-dpm}}{\mu\text{g Pi}}$	$\frac{^{3}\text{H-dpm}}{\mu\text{g Pi}}$	$\frac{^{14}\text{C-dpm}}{\mu\text{g Pi}}$
Control	4	158 ± 58	45 ± 27	130 ± 16	27 ± 4	394 ± 92	136 ± 90
	10	104 ± 59	24 ± 17	54 ± 15	10 ± 6	33 ± 21	8 ± 5
Naphthalene	4	214 ± 125	86 ± 20	104 ± 8	33 ± 7	819 ± 525	332 ± 111
	10	118 ± 26	26 ± 1	51 ± 6	8 ± 0	25 ± 2	5 ± 1
p-Cresol	4	268 ± 112	52 ± 29	142 ± 78	23 ± 23	669 ± 252	116 ± 95
	10	191 ± 49	36 ± 2	74 ± 62	11 ± 10	82 ± 65	16 ± 10
Naphthalene and p-cresol	4	124 ± 52	34 ± 4	66 ± 23	9 ± 3	478 ± 324	102 ± 23
	10	108 ± 18	24 ± 1	56 ± 19	10 ± 2	28 ± 18	7 ± 4

[a] Total phospholipid (PL) fractions of liver lipids were separated by TLC into phosphatidylethanolamines and phosphatidylcholines, as well as other components.

[b] Duplicate TLC fractions were used for determining contents of phosphorus in each phospholipid fraction.

EFFECTS OF 2,6-DIMETHYLNAPHTHALENE AND P-CRESOL ON THE MORPHOLOGY OF THE LIVER OF COHO SALMON

Methods

Coho salmon, each weighing an average of 30g(range of 25-34g), were obtained from Domsea Farms, Scatter Creek, WA. They were acclimated to laboratory holding tanks for two months. The stock supply of salmon was fed Oregon Moist Pellet (Hublou, 1963) and was maintained in flowing dechlorinated Seattle City water that slowly increased in temperature from 16.5°C at the beginning of the experiment to 19.5°C at the termination. Initially, 120 salmon were anesthetized, weighed, measured, and separated into 6 groups of 20 fish, none of which were fed. Three times per week each group of salmon was anesthetized with MS-222(ethyl m-aminobenzoate) and injected intraperitoneally with 0.05 ml of sockeye salmon (O. nerka) oil containing one of the following contaminants: 0.30mg 2,6-dimethylnaphthalene (DMN), 0.60mg 2,6-DMN, 0.30mg p-cresol, 0.60mg p-cresol, and 0.30mg 2,6-DMN in combination with 0.30mg p-cresol. The dose to each fish was either 10 or 20ppm(mg/kg) of contaminants. A control group was injected with salmon oil in the same manner but without any contaminant. Five fish from each group were sampled after a 5-wk exposure, and the remaining salmon were sacrificed after an additional 3-wk depuration period during which they received neither injections nor food.

The sampling procedure was the same for all fish: they were anesthetized, weighed, measured, killed by severing the spinal cord, and examined to determine the sex and the general condition of the external and internal organs. Portions of tissue from the tip of the posterior lobe of the liver were excised and placed in a fixative solution containing 0.7% glutaraldehyde, 3% formalin, and 0.5% acrolein in sodium cacodylate buffer (0.1M sodium cacodylate, 0.02% $CaCl_2.H_2O$, 5.5% sucrose pH7.4) (Hawkes, 1974). After a cacodylate buffer wash the tissues were post-fixed in 1% osmium tetroxide in buffer. Dehydrating with ethanol and embedding in Spurr's medium (Spurr, 1969) completed the preparation of the tissue for sectioning with either glass or diamond knives. Semi-thin sections (1.0 μm) were stained with Richardson's stain for light microscopic examination. Thin sections were successively stained with lead citrate, uranyl acetate and again with lead citrate for electron microscopy. The stained sections were coded such that the sources of samples were unknown to an independent observer.

Results

After five week's exposure, fish in all groups including the controls seemed normal in behavior and general appearance. The average lengths and weights were the same in all groups at the end of the experiment as at the beginning and there were few mortalities.

Liver Morphology of 5-Week Exposure Group--

The livers of five fish in each group were examined by both light microscopy and transmission electron microscopy (Table 39; Figs. 22, 23). Sections of livers from starved control fish and fed control (from the stock supply) fish were compared and the liver morphology was normal in both groups except that there were fewer glycogen deposits and very few lipid vacuoles in the starved fish. Hepatocytes in all control salmon had a well developed microvillar surface facing the sinusoids (Fig. 24), and there were numerous polyribosomes and whorls of granular endoplasmic reticulum in the cytoplasm. Also, within the cytoplasm, there were numerous mitochondria, particularly in the portion of the cell adjacent to the space of Disse, as well as numerous lysosomal vesicles distributed throughout the hepatocytes. The nuclei typically occupied a major proportion of the hepatocytes and appeared to have a normal distribution of condensed chromatin (Fig. 25).

The liver morphology of many of the fish exposed to 10 ppm 2,6-DMN for five weeks appeared normal; however, forty percent had some damage to the endothelial cells and to the surface membranes of the hepatocytes facing the sinusoids (Table 39; Figs. 26, 27). Cytoplasmic organelles such as the mitochodria appeared normal but the cytoplasm in 40% of the fish was vacoulated and cytosol coagulation was evident in all the fish. In the group exposed to 20 ppm 2,6-DMN, all of the five fish examined had severely damaged sinusoidal borders (Table 39; Figs. 28, 29) and the endothelial cells were disrupted. In some cases, only fragments of the cell surface membranes remained and the space of Disse was no longer distinguishable. The cytosol of hepatocytes from this group of animals appeared coagulated (Table 39). Eighty percent of the livers from this group had clusters of necrotic cells with marginated chromatin in the nuclei and an equal number were found with vacuolated cytoplasm.

Damage to the sinusoidal borders was noted in all of the fish treated with 10 ppm p-cresol (Table 39; Figs. 30, 31): the endothelial cells and membranes of the adjacent portions of the hepatocytes were disrupted with consequent loss of the space of Disse. The cytoplasm of the hepatocytes in this group was quite different from the cytoplasm of either the 10 ppm or the 20 ppm 2,6-DMN treated groups. The configuration of the endoplasmic reticulum near the damaged sinusoidal borders was unusual: there were short stacks of granular endoplasmic reticulum interspersed in the cytosol in 75% of the fish treated with 10 ppm cresol and in 80% of the fish treated with 20 ppm cresol (Table 39). All salmon in the group treated for five weeks with 20 ppm p-cresol showed sinusoidal damage (Table 39; Figs. 32, 33). In many of these fish, the sinusoids were nearly occluded, border membranes were indistinguishable and there was no evidence of the space of Disse. The arrangement of the endoplasmic reticulum was similar to the 10 ppm p-cresol group with short fragments of the membranes occurring throughout the cells (Fig. 33), and there were vacuoles in the cytoplasm, although the cytosol did not appear coagulated.

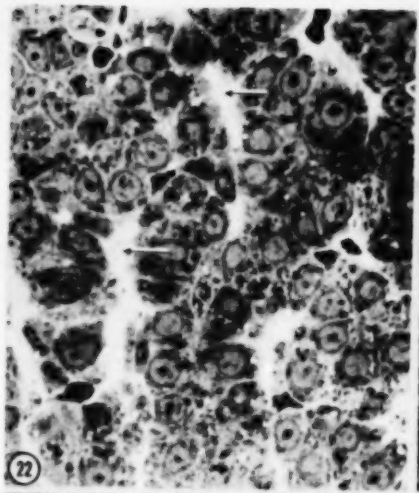
TABLE 39. PERCENT COHO SALMON WITH MORPHOLOGICAL ABNORMALITIES IN LIVER TISSUE AFTER EXPOSURE TO 2,6-DIMETHYLNAPHTHALENE (DMN) AND p-CRESOL[a]

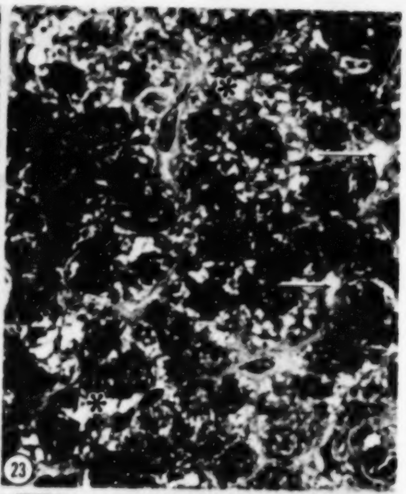
Exposure	Sinusoidal border damage		Marginated chromatin		Vacuolated chtoplasm		Coagulated cytosol	Fragmented Endoplasmic Reticulum
0	0[b]	0[c]	0[b]	0[c]	0[b]	0[c]	0[b]	0[b]
10 ppm 2,6-DMN	40	100	20	40	40	60	60	0
20 ppm 2,6-DMN	100	100	80	20	80	40	100	0
10 ppm p-Cresol	100	100	20	0	0	0	40	80
20 ppm p-Cresol	100	80	40	60	100	60	20	80
10 ppm DMN + 10 ppm p-Cresol	100	100	40	60	40	40	100	100

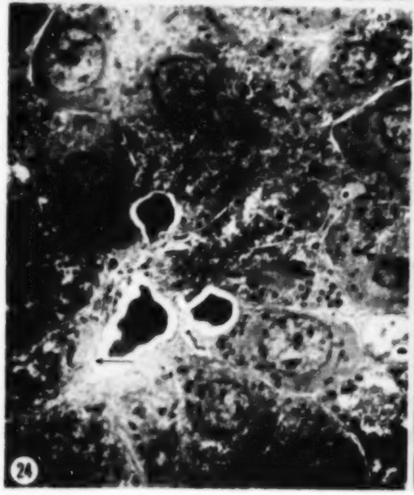
[a] Data based on five fish in each group

[b] After 5-week exposure

[c] After 3-week depuration in addition to the previous 5-week exposure







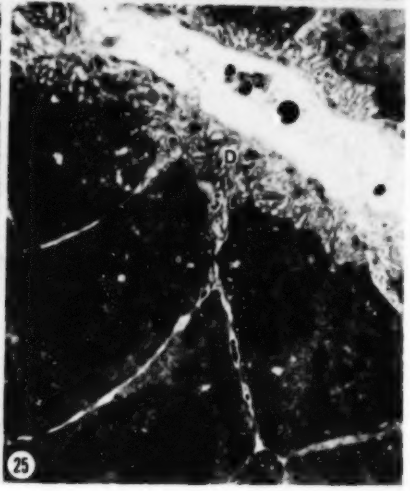
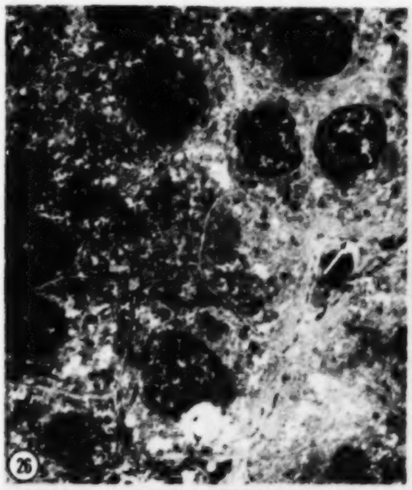


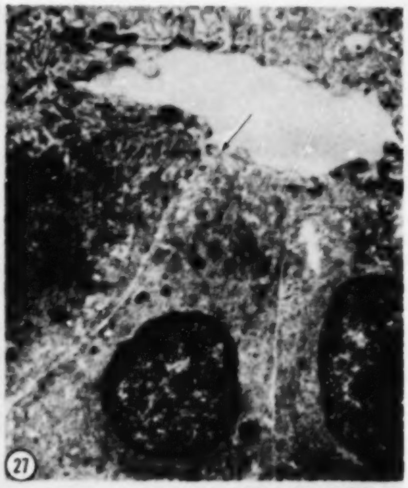
FIGURE 22. Hepatocytes from control coho salmon held for five weeks. Sinusoidal space (arrows). 680X.

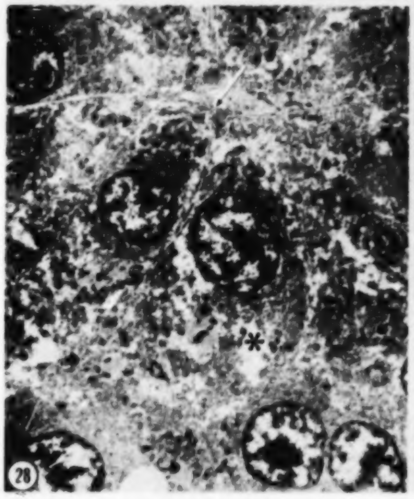
FIGURE 23. Hepatocytes from coho salmon exposed to 10 ppm 2,6-dimethylnaphthalene plus 10 ppm p-cresol for five weeks. Sinusoidal space (arrows); Vacuolated cytoplasm (*). 850X.

FIGURE 24. Hepatocytes from control coho salmon held for five weeks. Sinusoidal border (arrow). 2,400X.

FIGURE 25. Hepatocytes from control coho salmon held for five weeks. Space of Disse (D). 5,600X.







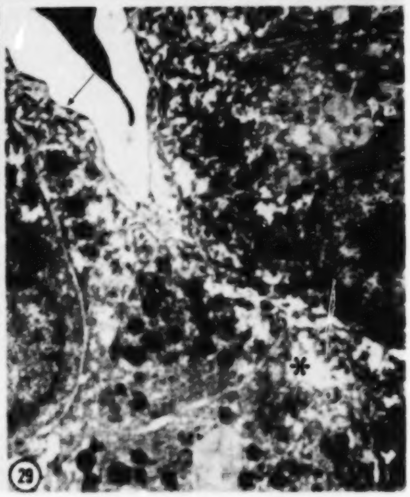
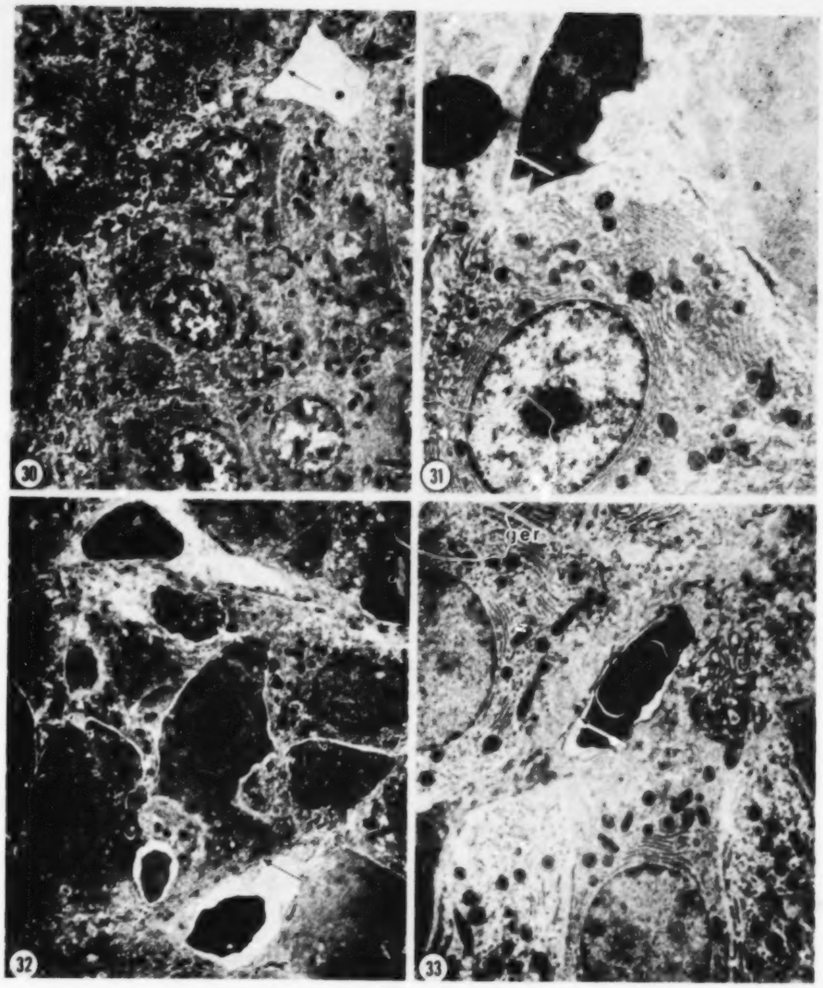


FIGURE 26. Hepatocytes from coho salmon after five weeks exposure to 10 ppm 2,6-dimethylnaphthalene. Sinusoidal border (arrow). 2,900X.

FIGURE 27. Hepatocytes from coho salmon after five weeks exposure to 10 ppm 2,6-dimethylnaphthalene. Sinusoidal border (arrow). 6,000X.

FIGURE 28. Hepatocytes from coho salmon exposed to 20 ppm 2,6-dimethylnaphthalene for five weeks. Sinusoidal border (arrow); Vacuolated cytoplasm (*). 3,000X.

FIGURE 29. Hepatocytes from coho salmon exposed to 20 ppm 2,6-dimethylnaphthalene for five weeks. Sinusoidal border (arrow); Vacuolated cytoplasm (*). 6,750X.



FIGURES 30-33. Hepatocytes from coho salmon after five weeks exposure to 10 ppm or 20 ppm p-cresol.
FIG. 30. Exposures to 10 ppm p-cresol. Sinusoidal border (arrow). 2,800X. FIG. 31. Exposure to 10 ppm p-cresol. Sinusoidal border (arrow). 6,000X. FIG. 32. Exposure to 20 ppm p-cresol. Sinusoidal border (arrow). 2,800X. FIG. 33. Exposure to 20 ppm p-cresol. Sinusoidal border (arrow); granular endoplasmic reticulum (ger). 5,800X.

Livers from all the salmon treated with 10 ppm 2,6-DMN in combination with 10 ppm p-cresol were found to have damaged sinusoidal borders (Table 39; Figs. 34, 35); however, in some of the fish, portions of the space of Disse could be distinguished. Many sinusoids were partially occluded. The cytoplasm of the hepatocytes in the combined group underwent similar changes to those seen in the groups treated with the single contaminants; the cytoplasm was coagulated and appeared similar to the cytoplasm in the hepatocytes of the 2,6-DMN treated group. The unusual arrays of short portions of the endoplasmic reticulum were observed in the region of the hepatocytes adjacent to the sinusoids, as was observed in the p-cresol treated groups.

Liver Morphology of 3-Week Depurated Groups--

Liver tissues from some groups appeared to have recovered slightly and in others become slightly more necrotic (Table 39), but the types of morphological changes in the liver tissue were the same as in the 5-wk-exposed groups. The incidence of sinusoidal damage remained high (80-100%) in all exposed groups, and remained 0 in the control.

Discussion

Disruption of the sinusoids of the liver of salmon exposed to either p-cresol or 2,6-DMN or both contaminants included loss of the endothelial cell lining of the sinusoids, loss of the microvillar portion of the hepatocytes, and compression of the perisinusoidal spaces (spaces of Disse). In some tissue sections, the lumina of the sinusoids were also compressed and appeared occluded. Similar sinusoidal changes have been reported in the liver tissue of experimental animals (primarily rats) and in humans that were chronically exposed to toxic substances (Tanikawa, 1979). In experiments conducted to determine the acute responses of rats to carbon tetrachloride, the same type of sinusoidal damage occurred within 30-120 minutes after exposure (Motta et al., 1978). This change, indicative of ischemia in the liver, has also been reported in the mummichog, _Fundulus heteroclitus_, chronically exposed to naphthalene (DiMichele and Taylor, 1978). In many examples of toxic injury to the liver, sinusoidal damage was the first pathologic change observed and this was postulated to result in the restriction of exchange of respiratory gases and nutrients, leading to hypoxia and further damage to liver cells (Miyai, 1979). Necrotic changes within the salmon hepatocytes, such as vacuolated cytoplasm and chromatin margination may, therefore, not only be related to direct toxic effects of the contaminants or their metabolites, but to a combination of toxicity and hypoxia.

Microscopic evidence indicated that 2,6-DMN and p-cresol affected the morphology of different cytoplasmic components of the liver cells. The changes in the endoplasmic reticulum in the hepatocytes from the p-cresol groups resembled that configuration reported in regenerating liver cells, rapidly proliferating cells, and pre-neoplastic hepatocytes (Miyai, 1979), but those changes were not observed in the liver cells of salmon treated with 2,6-DMN. Salmon from groups exposed simultaneously

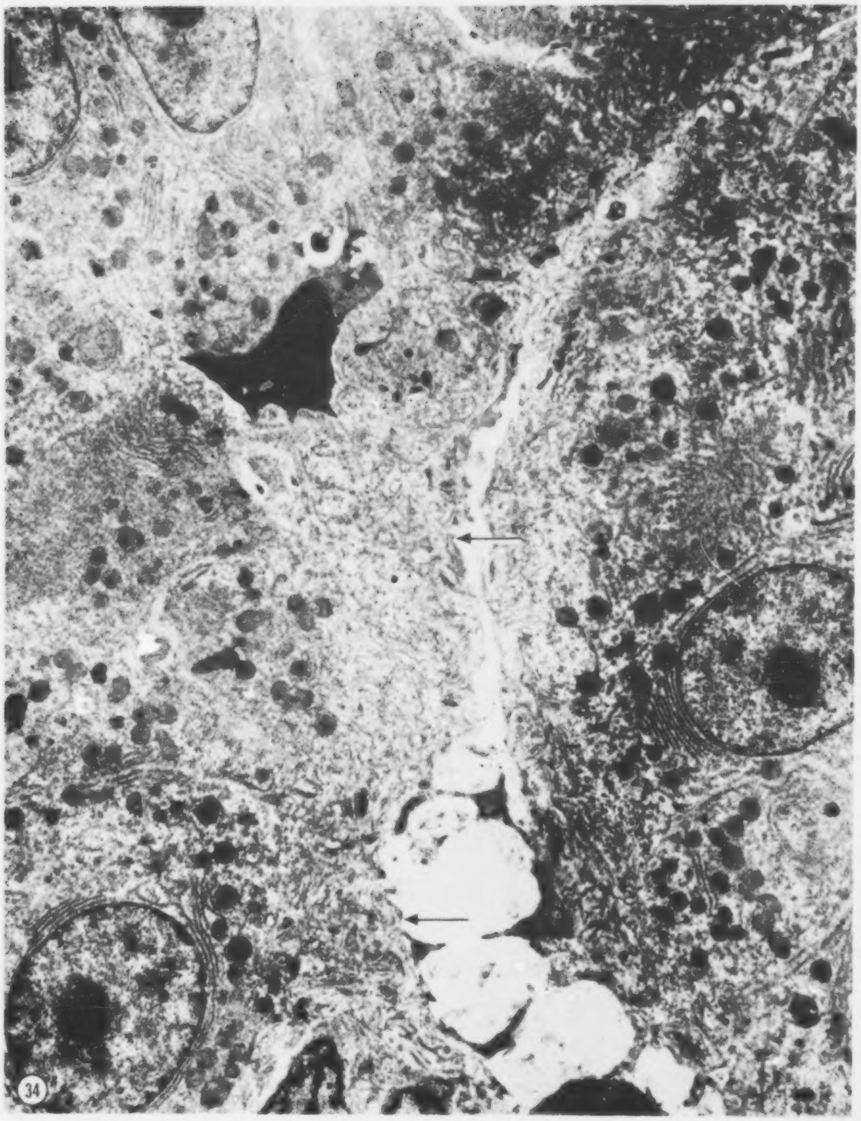


FIGURE 34. Hepatocytes from coho salmon exposed to 10 ppm 2,6-dimethylnaphthalene and 10 ppm p-cresol for five weeks. Sinusoidal border (arrow). 6,600X.

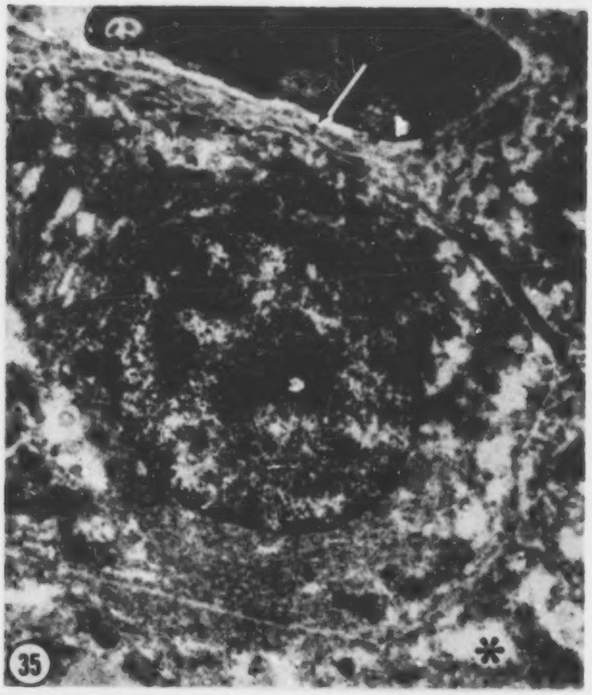


FIGURE 35. Hepatocytes from coho salmon exposed to 10 ppm of 2,6-dimethylnaphthalene and 10 ppm of p-cresol for five weeks. Sinusoidal border (arrow); Vacuolated cytoplasm (*). 5,600X.

to both contaminants seemed to have a combination of cytopathology reflecting both contaminants. The sinusoidal damage was similar to that of the groups exposed to the highest doses of single contaminants and was also similar to numerous cases of nonspecific toxic injury of the liver (Miyai, 1979). The hepatocellular changes, however, appeared to be a combination of cytosol coagulation, similar to that observed in the 2,6-DMN treated groups, and fragmentation of the endoplasmic reticulum, as in the p-cresol treated groups. This pattern of subcellular changes suggests that the contaminants interact with different cytoplasmic components.

Summary

We have demonstrated similar morphological changes in liver sinusoids of coho salmon exposed to p-cresol or 2,6-DMN. In addition, there are suggestions of specific types of hepatocellular changes distinctive to each contaminant. When the contaminants were administered in combination, cytopathological changes reflective of both contaminants were observed. Therefore, the contaminants appeared to affect the same hepatocytic organelles whether the exposure was to the individual or combined compounds. Although we have no direct measure of liver impairment as a result of the contaminant exposures, the morphological changes, particularly those involving the sinusoids, were similar to hepatic changes seen in other organisms with functionally detrimental conditions such as hepatitis, toxic injury, and hypoxia (Tanikawa, 1979).

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